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ON STEROID NUCLEOTIDES

II. SYNTHESIS OF Δ5-3β-HYDROXY-STEROID NUCLEOTIDES

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SUMMARY

5-androsten-3β-ol-17-one-ADP, 5-pregnen-3β-ol-20-one-ADP, and 5-pregnene-3β,17α-diol-20-one-ADP have been prepared from Δ5-3β-hydroxy-steroid-phosphoric acid and the dicyclohexylguanidinium salt of AMP-phosphoramidate. The synthesis of 5-androsten-3β-ol-17-one-ATP was achieved by condensation of 5-androsten-3β-ol-17-one-phosphoric acid and ADP, using dicyclohexylcarbodiimide as condensing agent.

After purification by column chromatography, paper chromatography in two solvent systems and paper electrophoresis, the Δ5-3β-hydroxy-steroid nucleotides were found to be relatively unstable.

INTRODUCTION

The *in vivo* conversion of 5-pregnen-3β-ol-20-one (5-pregnenolone) and 5-pregnene-3β,17α-diol-20-one (17α-hydroxy-5-pregnenolone) to plasma 5-androsten-3β-ol-17-one (dehydroepiandrosterone) in the dog has been previously reported[1]. Approximately 70% of the total dehydroepiandrosterone formed was conjugated in such a way that it could be liberated by incubation with commercial alkaline phosphatase. Furthermore, the isolation from peripheral human blood plasma of dehydroepiandrosterone conjugates containing phosphoric acid has already been described[2]. These data may reflect the possible existence of hitherto unknown steroid conjugates, such as steroid nucleotides. The preparation of dehydroepiandrosterone nucleotides has been reported in a preliminary communication[3]. The present paper contains a detailed description of the synthesis of dehydroepiandrosterone-ADP and dehydroepiandrosterone-ATP as well as preparation of 5-pregnenolone-ADP and 17α-hydroxy-5-pregnenolone-ADP.

RESULTS AND DISCUSSION

Steroid-ADP compounds were prepared from steroid phosphates and the dicyclohexylguanidinium salt of AMP in dry pyridine in analogy to the synthesis of phenyl-ADP and flavine-adenine dinucleotide[4]. Dehydroepiandrosterone-ATP was obtained

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Abbreviations: AMP, adenosine-5′-phosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate.

by condensation of dehydroepiandrosterone-phosphoric acid[5] and ADP in 75% pyridine, employing dicyclohexylcarbodiimide as the condensing agent as in the preparation of amino acid adenylates[6]. The crude reaction products were submitted to a preliminary chromatography on Dowex 2 ion exchange resin (formate form)[7]. The steroid nucleotides were eluted by 0.001 *N* formic acid while the dicyclohexylguanidinium salt of AMP and AMP itself were removed by 0.01 *N* and 0.1 *N* formic acid respectively (Fig. 1). The compounds were purified further by chromatography on paper using the solvent systems *n*-butanol–1 *N* NH_4OH, a modified BUSH system[8] and isobutyric acid–0.1 *M* Versene–1 *N* NH_4OH[9]. The location of $\Delta 5$-3β-hydroxysteroid nucleotides on paper strips was determined by u.v. absorption, and either a color reaction with sulfuric acid–ethanol characteristic for the $\Delta 5$-3β-hydroxy-configuration of steroids[10] or the ZIMMERMANN reaction of 17-ketosteroids[11]. Table I

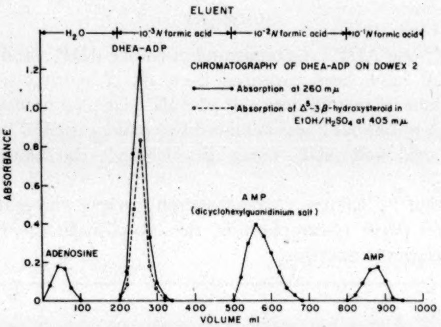

Fig. 1. Column chromatography of 5-androsten-3β-ol-17-one-ADP on Dowex-2 ion exchange resin (formate form).

TABLE I

R_F VALUES OF STEROID NUCLEOTIDES, NUCLEOTIDES AND STARTING MATERIAL IN DIFFERENT SYSTEMS OF PAPER CHROMATOGRAPHY

Compound	*n-butanol–1 N NH_4OH*	*Isobutyric acid–0.1 M Versene–1 N NH_4OH (100:1.6:60 v/v/v)*
Steroid nucleotides		
5-androsten-3β-ol-17-one-ADP	0.40	0.79
5-pregnen-3β-ol-20-one-ADP	0.48	0.81
5-pregnene-3β,17α-diol-20-one-ADP	0.35	0.77
5-androsten-3β-ol-17-one-ATP	0.32	0.76
Starting materials		
5-androsten-3β-ol-17-one-phosphoric acid	0.72	1.00
5-pregnen-3β-ol-20-one-phosphoric acid	0.81	1.00
5-pregnene-3β,17α-diol-20-one-phosphoric acid	0.51	1.00
AMP	0.00	0.63
ADP	0.00	0.46
ATP	0.00	0.28
AMP-phosphoramidate, dicyclohexylguanidinium salt	0.27	0.70

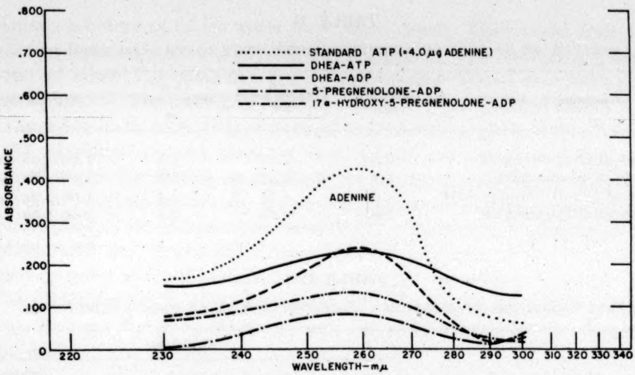

Fig. 2. Analysis of steroid nucleotides eluted from paper: absorption curves of adenine components (Table II).

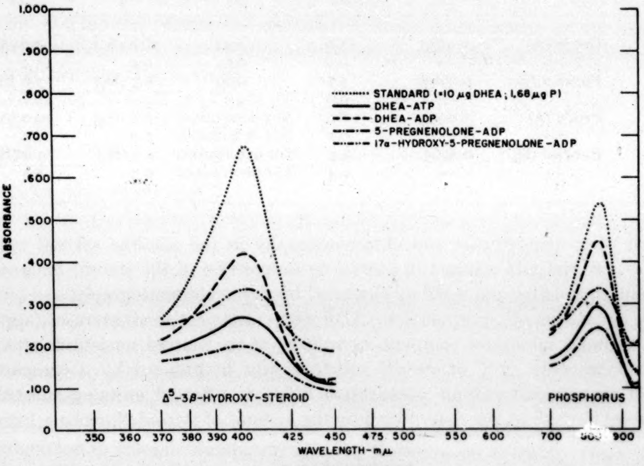

Fig. 3. Analysis of steroid nucleotides eluted from paper: absorption curves of Δ5-3β-hydroxysteroids (in ethanol–sulfuric acid reagent[10]) and of phosphate (after color reaction with ammonium molybdate[12]) (Table II).

gives the R_F values of the steroid nucleotides as well as of nucleotides and starting material. Aliquots of 3β-hydroxy-steroid nucleotides were analysed for adenine using the u.v. absorption at 260 mμ and submitted to micro-analysis for phosphorus[12] and the sulfuric acid–ethanol reaction for Δ5-3β-hydroxy steroids (Figs. 2 and 3). Results of such experiments are seen in Table II.

After paper electrophoresis in acetate buffer and repeated paper chromatography in the solvent systems already described, the purified Δ5-3β-hydroxy-steroid nucleotides were found to be uniform, although the pure compounds were relatively unstable.

TABLE II

ANALYSIS OF STEROID NUCLEOTIDES ELUTED FROM PAPER (Figs 2 and 3)

Compound	*Adenine μg*	*Phosphoric acid μg*	*Δ5-3β-hydroxy-steroid μg*	*Molecular ratio*
5-androsten-3β-ol-17-one-ADP	2.6	1.27	5.5	1.00:2.08:0.97
5-pregnen-3β-ol-20-one-ADP	1.1	0.53	2.6	1.00:2.10:1.01
5-pregnene-3β,17α-diol-20-one-ADP	1.1	0.51	2.5	1.00:2.02:0.94
5-androsten-3β-ol-17-one-ATP	1.0	0.68	2.2	1.00:3.09:1.03

TABLE III

ENZYMIC HYDROLYSIS OF 5-PREGNEN-3β-OL-20-ONE-ADP BY PHOSPHODIESTERASE

Substrate μg	*Enzyme*	*Units*	*Found after*			
			Enzymic hydrolysis			*Subsequent acid hydrolysis*
			Steroid μg	*Phosphorus μg*	*Steroid phosphate μg*	*Steroid μg*
34.5	Crude (A)	0.0055	15.0	1.44	18.8 (A_1)	0.0 (A_2)
34.5	—	—	0.0	0.0	0.0	15.2
34.5	Purified (B)	0.0063	5.5	0.69	6.9 (B_1)	7.6 (B_2)
34.5	—	—	0.0	0.0	0.0	15.1
20.5	Crude (A)	0.0055	8.6	Not determined	10.8 (A_1)	0.0 (A_2)
20.5	—	—	0.2	Not determined	0.3	8.5
20.5	Purified (B)	0.0063	3.4	Not determined	4.3 (B_1)	4.9 (B_2)
20.5	—	—	0.4	Not determined	0.5	8.3

Storage at room temperature and chromatography in the alkaline solvent system *n*-butanol–1 *N* NH_4OH resulted in partial decomposition of the steroid nucleotides into steroid phosphates and AMP as identified by paper chromatography.

After incubation of 5-pregnenolone-ADP with crude phosphodiesterase* (approx. $1.5 \cdot 10^{-4}$ units/μg substrate) complete hydrolysis of the steroid nucleotide was observed, whereas only 37 % of steroid substrate was hydrolyzed by a comparable amount of the purified enzyme preparation (approx. $1.8 \cdot 10^{-4}$ units/μg substrate). The degree of hydrolysis was determined by the amount of steroid phosphate formed.

EXPERIMENTAL

5-androsten-3β-ol-17-one-phosphoric acid

The synthesis of this compound has been described in a previous publication[5].

5-pregnen-3β-ol-20-one-phosphoric acid

316 mg (1 mmole) 5-pregnen-3β-ol-20-one were dissolved in 15 ml dry pyridine and 100 ml dry ether. At —15°, 0.084 ml (0.9 mmole) phosphorus oxychloride in 25 ml ether were added over the course of 1 h with continuous stirring. The reaction mixture was kept for 2 h below 0° and subsequently at room temperature for an

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* Obtained through the courtesy of Dr. S. A. DICKMAN, University of Utah, Salt Lake City, Utah (U.S.A.).

additional 2 h before 25 ml ice water were added slowly. After 2 h at room temperature, the two phases were separated and the aqueous layer extracted 3 times, each with 100 ml ether. The combined ether extracts were treated once with 50 ml 1 *N* sodium hydroxide. The combined alkaline solutions were acidified with 5 % sulfuric acid. The precipitate, consisting of 5-pregnen-3β-ol-20-one-phosphoric acid was separated by centrifugation and, after the supernatant had been decanted, was washed with 10 ml water. For further purification, the precipitate was dissolved in a minimal volume of 1 *N* sodium hydroxide, the undissolved material removed by filtration and the steroid phosphoric acid precipitated with 5 % sulfuric acid. 5-pregnen-3β-ol-20-one phosphoric acid was recrystallized from aqueous methanol or ethyl acetatehexane. The melting point of the dry material was 142–144°, the yield 163 mg (41 %).

Calculated for $C_{21}H_{33}O_5P$. H_2O (414.47): C, 60.84; H, 8.51; P, 7.47 %. Found: C, 60.53; H, 8.40; P, 7.52 %. Molecular ratio: Δ53-β-hydroxy-steroid: phosphoric acid = 1.00:0.98.

5-pregnene-3β,17α-diol-20-one-phosphoric acid

This was prepared from 332 mg (1 mmole) 5-pregnene-3β,17α-diol-20-one and 0.084 ml. (0.9 mmole) phosphorus oxychloride in the same manner as for 5-pregnen-3β-ol-20-one-phosphoric acid. Recrystallization yielded 150 mg of colorless crystals with m.p. 162–163° (36 % yield).

Calculated for $C_{21}H_{33}O_6P$ (412.45): C, 61.09; H, 8.00; P, 7.51 %. Found: C, 61.31; H, 8.24; P, 7.28 %. Molecular ratio: Δ5-3β-hydroxy-steroid: phosphoric acid = 1.00:0.96.

5-androsten-3β-ol-17-one-ADP

15 mg (0.025 mmole) of the dicyclohexylguanidinium salt of AMP-phosphoramidate and 92 mg (0.25 mmole) of 5-androsten-3β-ol-17-one-phosphoric acid were dried separately for 6 h at 80° *in vacuo* and subsequently dissolved in 25 ml dry pyridine and kept for 3 days at 40° in a sealed tube. After evaporation of the solvent *in vacuo* 25 ml water were added. After acidification the solution was filtered and the filtrate extracted three times with 25 ml ethyl acetate–*n*-butanol (1:1, v/v) in order to remove unreacted steroid phosphoric acid. The aqueous solution was evaporated *in vacuo* and the residue washed 3 times each, with 25 ml absolute ethanol, the supernatant being removed after centrifugation. The residue was dried *in vacuo* and submitted to column chromatography on Dowex-2 ion exchange resin (formate form)[7] as will be described. Recrystallization from methanol–water gave 11 mg (62 %) of colorless crystals. The pure compound did not possess a definite melting point.

Calculated for $C_{29}H_{41}O_{11}N_5P_2$ (697.63): P, 8.88 %. Found: P, 8.65 %. Molecular ratio: adenine: phosphoric acid: Δ5-3β-hydroxy-steroid = 1.00:2.06:0.96.

5-pregnen-3β-ol-20-one-ADP

This was prepared from 15 mg (0.025 mmole) dicyclohexylguanidinium salt of AMP phosphoramidate and 103 mg (0.25 mmole) 5-pregnen-3β-ol-20-one-phosphoric acid. After column chromatography 8.5 mg (48 %) of a colorless powder without definite melting point were obtained.

Calculated for $C_{31}H_{45}O_{11}N_5P_2$ (725.69): P, 8.54 %. Found: P, 8,31 %. Molecular ratio: adenine: phosphoric acid: Δ5-3β-hydroxy-steroid = 1.00:2.03:0.95.

5-pregnene-3β,17α-diol-20-one-ADP

This was prepared from 15 mg (0.025 mmole) AMP phosphoramidate-dicyclohexylguanidinium salt and 103 mg (0.25 mmole) 5-pregnene-3β,17α-diol-20-one-phosphoric acid as described. 9.3 mg of colorless powder without definite melting point were obtained (yield: 49 %).

Calculated for $C_{31}H_{45}O_{12}N_5P_2$ (741.69): P, 8.35 %. Found: P, 8.27 %. Molecular ratio: adenine: phosphoric acid: Δ5-3β-hydroxy-steroid = 1.00:2.04:0.97.

5-androsten-3β-ol-17-one-ATP

85 mg (0.2 mmole) ADP and 72 mg (0.2 mmole) 5-androstene-3β-ol-17-one-phosphoric acid were dissolved in 10 ml 70 % pyridine. At 5° a solution of 1 g dicyclohexylcarbodiimide in 1 ml dry pyridine was added. After 24 h of continuous stirring at 5°, the reaction mixture was kept for an additional 24 h at room temperature. The precipitate was collected by filtration and washed with 5 ml 70 % pyridine, and the filtrate diluted with 50 ml water. This aqueous solution was kept at room temperature for 1 h, filtered and the filtrate evaporated to dryness *in vacuo*. The residue was treated twice with 20 ml acetone and twice with 20 ml absolute ethanol, dried *in vacuo* and purified by column chromatography. 17 mg (11 %) of a colorless powder without definite melting point were obtained.

Calculated for $C_{29}H_{42}O_{14}N_5P_3$ (777.59): P, 11.96. Found: P, 11.64. Molecular ratio: adenine: phosphoric acid: Δ5-3β-hydroxy-steroid = 1.00:3.07:0.93.

All melting points reported were determined under a microscope and are not corrected. All elemental analyses were performed by Elec. Microanalytical Laboratories, Los Angeles, Calif. (U.S.A.).

Column chromatography of steroid nucleotides

The crude material was dissolved in a minimum amount of water and applied to a Dowex-2 ion exchange column (formate form), measuring 5 cm in height and 0.5 cm diameter. 200–300 ml of water, 300 ml of 0.001 *N*, 300 ml of 0.01 *N* and 300 ml of 0.1 *N* formic acid were used as eluents, the volume of the individual sub-fractions being 20 ml and the flow rate approximately 2 ml/min. Each individual fraction eluted from the column was assayed for adenine, employing u.v. absorption at 260 mμ. An aliquot of each fraction containing adenine was evaporated *in vacuo* and the residue submitted to the color reaction with ethanol–sulfuric acid (1:2, v/v) characteristic for Δ5-3β-hydroxy-steroids. As shown in Fig. 1, steroid nucleotides were eluted with 0.001 *N* formic acid. All fractions containing adenine and Δ5-3β-hydroxy-steroids were combined and evaporated to dryness *in vacuo*.

Paper chromatography of steroid nucleotides

Two solvent systems were used for paper chromatography of steroid nucleotides: *n*-butanol–1 *N* NH_4OH[8] and isobutyric acid–0.1 *M* Versene–*N* NH_4OH (100:1.6:60 v/v/v)[9]. After ascending paper chromatography in 50 % methanol[14] essentially as described in a previous publication[14] descending paper chromatography was performed. For paper chromatography in the solvent system *n*-butanol–1 *N* NH_4OH, 6 h equilibration was allowed prior to development of the paper chromatogram with the mobile phase. The chromatograms were run to the front, which required between 18 and 20 h for both systems. The spots containing steroid nucleotides were located by u.v. absorption and either the **ZIMMERMANN** reaction indicative of 17-ketosteroids or the ethanol–sulfuric acid reaction. The steroid nucleotides finally were eluted using 50 % methanol and evaporated to dryness *in vacuo*.

Enzymic hydrolysis of 5-pregnen-3β-ol-20-one-ADP

34.5 μg 5-pregnen-3β-ol-20-one-ADP dissolved in 0.1 ml 0.25 *M* Na-succinate–HCl-buffer of pH 6.5 were pipetted into each of four graduated centrifuge tubes. To 2 of the tubes 0.01 ml of a crude phosphodiesterase preparation[13] (= A) containing approx. 5.5 units/ml were added and 0.01 ml of the purified phosphodiesterase preparation (= B) containing 6.3 units/ml to the remaining two tubes. All samples, including controls with buffer and enzyme were diluted to 0.2 ml and incubated at 37° for 1 h. Following the incubation, 10 ml of water was pipetted into each tube. All samples were acidified to pH 3 using 1 *N* sulfuric acid and extracted immediately 3 times with 10 ml ethyl acetate–butanol (1:1, v/v). In order to hydrolyze pregnen-3β-ol-20-one-ADP, not cleaved during incubation, 1 ml concentrated sulfuric acid was added to the aqueous phase of each sample. After 30 min in a boiling water bath all samples were extracted 3 times with 10 ml ethyl acetate. The combined ethyl acetate–butanol extracts (A_1 and B_1) containing 5-pregnen-3β-ol-20-one-phosphate were washed once with 10 ml water and the combined ethyl acetate extracts (A_2 and B_2) containing free 5-pregnen-3β-ol-20-one once with 10 ml 1 *N* sodium hydroxide and twice with 10 ml water. All extracts were evaporated, the residues dissolved in methanol and aliquots assayed for Δ5-3β-hydroxy-steroids, using the control extracts as blanks. Additional aliquots of A_1 and B_1 were analyzed for phosphorus. The remaining material of all extracts was chromatographed on paper: A_1 and B_1 with the appropriate controls in *n*-butanol–1 *N* NH_4OH, A_2 and B_2 with controls in the solvent system propylene glycol–methyl cyclohexane[15] which enabled the isolation of 5-pregnenolone.

Paper electrophoresis

Paper electrophoresis was performed using 0.05 *M* acetate buffer (pH 5.6). At a current of 5 mA for 16 h at 25°, the mobility of the steroid nucleotides to the anode ranged between 7.3 and 10.3 cm. The spots were located by u.v. absorption and the color reactions previously described; these spots were eluted and the eluates assayed for adenine and steroid.

ACKNOWLEDGEMENT

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THE GLOBULIN OF CALF THYMUS NUCLEI AND THE *IN VITRO* INCORPORATION OF [^{14}C]ADENOSINETRIPHOSPHATE INTO GLOBULIN-RNA

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SUMMARY

Investigation of the globulin fraction of isolated thymus nuclei has shown that the globulin contains RNA and is a polydisperse ribonucleoprotein complex. The globulin fraction is able to incorporate [8-^{14}C]ATP into its RNA moiety. Partial fractionation of the globulin reveals that the fractions have different rates of [^{14}C]ATP incorporation.

INTRODUCTION

The ability of isolated cell nuclei to incorporate nucleic acid precursors *in vitro* has been demonstrated by a number of investigators in calf thymus, and in rabbit liver and appendix[1–4]. These precursors have been shown to be incorporated into the nucleic acids and are closely related to protein synthesis[1]. Recently a "pH 5 enzyme" has also been found in isolated nuclei from calf thymus and liver[5,6], and chicken kidney[6] which catalyzes pyrophosphate–ATP exchange in the presence of amino acids, thus being similar to the "pH 5 enzyme" prepared from cytoplasmic supernatant by Zamecnik and others[7,8]. These findings, therefore, substantiate the belief that RNA plays an important role in the metabolic processes of the cell nucleus. In the light of these studies, further examination of the relationship between nuclear RNA and nuclear protein fractions would appear to be of interest.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; AMP, adenosine monophosphate; CTP, cytosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; RNase, ribonuclease.

RNA has been found to be present in a number of protein fractions isolated from cell nuclei. ZBARSKII AND GEORGIEV reported that in isolated rat liver nuclei both globulin and lipoprotein contain RNA[9]; from thymus nuclei, ALLFREY AND MIRSKY obtained a pH 7.1-soluble fraction containing RNA which was metabolically active[10]; in addition, there is the "pH 5 enzyme" prepared by HOPKINS *et al.*[5,6]. Since lipoprotein can only be prepared from an alkaline extract of the nuclei[11], it seems that the globulin, which is prepared by mild procedure[12], would be best suited for investigation. This communication summarizes the results obtained from the studies of the nuclear globulin, demonstrating that the RNA moiety is an integral part of the globulin. It will also show that the globulin fraction incorporates ATP into its RNA moiety.

MATERIALS AND METHODS

Fresh calf thymus was obtained from a local packing house immediately after the animal was killed and nuclei were prepared according to ALLFREY[13]. Microscopic examinations showed that the preparations were quite clean, with very little non-nuclear contamination.

For the preparation of the globulin, the nuclei were extracted with 0.14 *M* NaCl which had been adjusted to pH 7.0. The extraction was carried out by first homogenizing the nuclei with 0.14 *M* NaCl in a Servall Omni-mixer for 15 sec in an ice–water bath, and then by stirring it for 1 h in the cold. After centrifuging the homogenate at 15,000 × *g* for 15 min, the supernatant was collected. The extraction procedure was repeated twice, the combined extract was subjected to 20,000 × *g* for 30 min, and the supernatant collected. Acidification of the opalescent solution to pH 5.0–5.2 with *N* acetic acid gave a precipitate which was then dissolved in 0.05 *M* Tris–0.14 *M* NaCl, pH 8.5.

Purification of the globulin was accomplished by repeating the isoelectric precipitation three times. For incubation studies, the precipitate was dissolved in Medium A according to HECHT *et al.*[8]. After three more times of reprecipitation, the preparation in Medium A was stored frozen, until use.

[8-^{14}C]ATP was obtained from Schwartz Laboratory. ATP, CTP, phosphoenolpyruvate and pyruvic kinase were purchased from Sigma Chemical Company.

RNA was prepared from the globulin by phenol extraction[14]. The nucleotide composition of RNA was analyzed by alkaline hydrolysis and paper electrophoresis as described by MARKHAM AND SMITH[15]. RNA content was measured by the orcinol method[16] and u.v. spectrophotometry; the two procedures gave results that checked to within 3 %. To test for DNA, the diphenylamine method[16] was used, in addition to the test for thymidylic acid from its nucleotide composition. Protein concentration was determined by Folin procedure[17].

Sedimentation patterns were taken with a Spinco Model E Ultracentrifuge.

The incubation conditions are given in the legends to the tables and figures. Reactions were stopped by chilling the incubating mixture in an ice–water bath, and an equal volume of cold 20 % TCA was added. The precipitate, after centrifugation, was washed 5–7 times with 7-ml portions of cold 10 % TCA, twice with cold 95 % ethanol, and twice with ether. The dried precipitate was taken up in NH_4OH and plated on stainless steel planchets for counting in a Tracerlab windowless gas flow counter.

RESULTS

Globulin as a ribonucleoprotein

The globulin preparations obtained from isolated calf thymus nuclei always showed an absorption spectrum characteristic of a nucleoprotein. Fig. 1 is such an absorption spectrum which shows maximal and minimal absorptions at 259 mμ and 243 mμ, respectively. The preparations were found to be diphenylamine negative and free of thymidylic acid, indicating the absence of DNA. Determinations of RNA and of protein gave an RNA content of about 10 %. Analyses of the RNA revealed only adenylic, cytidylic, guanylic and uridylic acids as its constituents (Table I). These results confirm that the nucleic acid present in the globulin is RNA. Furthermore, the RNA content was quite constant from preparation to preparation, and was not affected by further repeated isoelectric precipitation.

The RNA present in the globulin was attacked by pancreatic RNase. Digestion

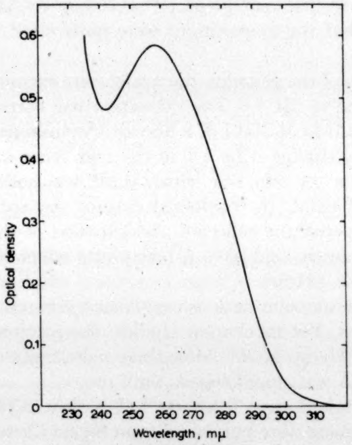

Fig. 1. U.V. absorption spectrum of nuclear globulin in saline-Tris buffer, pH 8.6.

TABLE I

COMPOSITION AND CONTENT OF RNA FRACTIONS OF CALF THYMUS NUCLEI

RNA fractions	*Molar nucleotide composition (Relative to adenylic acid as 10)*				*% of RNA*
	Adenylic	*Cytidylic*	*Guanylic*	*Uridylic*	
Globulin	10	18.3	15.6	12.2	10.8 ± 0.36(6)*
(Molar ratio)	17.8 ± 0.52	32.4 ± 0.98	27.8 ± 1.0	21.8 ± 0.43(5)*	
Whole nuclei**	10	14.2	16.2	8.5	
pH 7.1-soluble***	10	16.3	16.7	13.1	
"Nucleolar"***	10	15.2	16.7	12.9	

* Figures in parentheses indicate number of analyses on different preparations.
** Taken from V. G. ALLFREY AND A. E. MIRSKY[9].
*** Taken from V. G. ALLFREY AND A. E. MIRSKY[10].

of the whole globulin with RNase in the cold (4°) released about 75 % of the RNA as dialyzable degradation products. The action of RNase also precipitated irreversibly approx. 66 % of the protein. It appears that the protein moiety of the globulin maintains its secondary structure by conjugation with the RNA. The destruction of the polynucleotide structure apparently caused the collapse of the protein configuration, strongly suggesting that most of the globulin is ribonucleoprotein in nature.

The globulin is complex not only in that it contains RNA, but there is also considerable molecular heterogeneity. Fig. 2a shows an ultracentrifuge diagram of a supernatant solution of the globulin after centrifuging at 40,000 × *g* for 30 min.

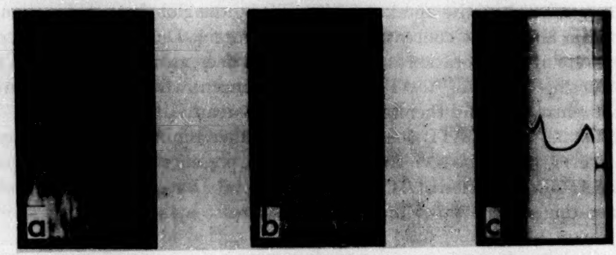

Fig. 2. Ultracentrifuge patterns of nuclear globulin in 0.14 *M* NaCl–0.05 *M* Tris, pH 8.5. (a) 40,000 × *g* supernatant of globulin solution, 96 min after attaining speed of 59,780 rev./min at 6.1°; bar angle 70°. (b) 40,000 × *g* supernatant of globulin solution, 170 min after attaining speed of 59,780 rev./min at 7.6°; bar angle 70°. (c) 40,000 × *g* pellet, 80 min after attaining speed of 24,630 rev./min at 6.9°; bar angle 65°.

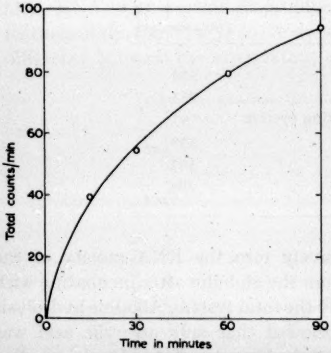

Fig. 3. Time course of incorporation of $[8\text{-}^{14}C]$ATP into globulin. The system contained: 6.5 mg of globulin; 2.5 µmoles $[8\text{-}^{14}C]$ATP ($3.4 \cdot 10^5$ counts/min/µmole); 25 µmoles phospho-enol-pyruvate; 75 µg pyruvic kinase; 2.5 µmoles CTP; 10 µmoles $MgCl_2$; 100 µmoles Tris, pH 7.5; and 750 µmoles sucrose; in a final volume of 2.5 ml. Aliquots of 0.5 ml were taken after incubation without shaking at 37° for the periods of time as indicated.

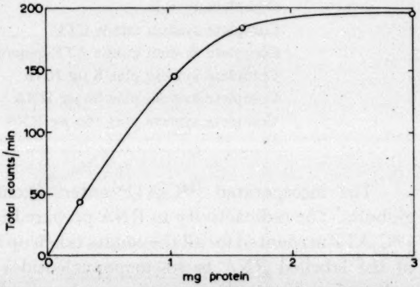

Fig. 4. Effect of protein concentration on the incorporation of $[8\text{-}^{14}C]$ATP into globulin. The system contained: 1 µmole $[8\text{-}^{14}C]$ATP ($3.4 \cdot 10^5$ counts/min); 10 µmoles phospho-enol-pyruvate; 30 µg pyruvic kinase; 1 µmole CTP; 4.0 µ moles $MgCl_2$; 40 µmoles Tris, pH 7.5; 250 µmoles sucrose; and amounts of globulin as indicated; in a final volume of 1 ml. The system was incubated without shaking for 90 min at 37°.

The slow component, after prolonged ultracentrifugation, shows further splitting as can be seen in Fig. 2b. The 40,000 × *g* pellet, when dissolved in saline-buffer, shows the rapidly sedimenting components (Fig. 2c). The use of 40,000 × *g* to clarify the solution was arbitrary; it simply makes the sedimentation pattern clearly visible. The effect of lack of transparency in the solution is apparent in Fig. 2c. It is obvious that the whole globulin fraction is a highly polydisperse system. However, the possibility cannot be ruled out that association–dissociation may have occurred during the course of preparation.

Incorporation of [8-^{14}C]ATP by globulin RNA

Figs. 3 and 4 show the uptake of [^{14}C]ATP by the globulin as a function of time of incubation and of the concentration of the protein. During the early incubating phase the rate of incorporation is rapid, and then decreases, but it does not level off. The relationship between [^{14}C]ATP uptake and concentration of the globulin is linear at low concentrations and thereafter reaches a plateau.

The uptake of [^{14}C]ATP does not require either the ATP-generating system or CTP. However, as can be seen from Table II, the presence of either or both enhances the incorporation. Omission of CTP reduces the uptake by 34 %, and the absence of an ATP-generating system causes the uptake to decrease by 40 %.

TABLE II

INCORPORATION OF [8-^{14}C]ATP INTO GLOBULIN RNA

The system contained: 3.2 mg of globulin; 1 µmole [8-^{14}C]ATP ($3.99 \cdot 10^5$ counts/min); 10 µmoles phospho-enol-pyruvate; 30 µg pyruvic kinase; 1 µmole CTP; 4.0 µmoles $MgCl_2$; 40 µmoles Tris, pH 7.5; and 250 µmoles sucrose; in a final volume of 1.0 ml. The system was incubated without shaking for 90 min at 37°.

	Total counts/min
Complete system	353
Complete system minus CTP	232
Complete system minus ATP-generating system	197
Complete system plus 8 µg RNA	330
Complete system plus 80 µg RNA	303
Complete system plus 160 µg RNA	289

The incorporated [^{14}C]ATP enters exclusively into the RNA moiety of the globulin. The radioactivity in RNA prepared from the globulin after incubating with [^{14}C]ATP accounted for all the counts taken up by the total system. Alkaline hydrolysis of the labelled RNA to its mononucleotides showed that only adenylic acid was radioactive. Under the conditions as described in the legend to Table II, the labelled adenylic acid represents 1.1 moles/1,000 moles of total AMP in the RNA.

It is apparent that the incorporation of [8-^{14}C]ATP is closely related to the RNA of the globulin. However, extra RNA, prepared from the globulin and added to the incubation system has no effect on the uptake of [^{14}C]ATP. This may also be a result of the close association of the RNA and protein portions of the globulin. Higher concentrations of added RNA even appear to have an inhibitory effect on the incorporation.

DISCUSSION

The nuclear globulin, prepared as described here, is not homogeneous but contains an almost continuous spectrum of molecular species. Centrifugation at 20,000 × *g* for 30 min was chosen only as an arbitrary standard to define the globulin fraction. It produces a preparation with a constant content of RNA. There also seems to be little doubt that the globulin contains RNA bound to protein. From the present study one cannot conclude, however, that all the molecular species in the globulin fraction are RNA-containing proteins. In fact, the RNA content must vary from one component to another, since it is found that the 40,000 × *g* pellet of the globulin has more RNA than the supernatant. It follows, therefore, that the nucleotide composition of the globulin RNA is merely an average of all the RNA components present in the globulin fraction. Nevertheless, it may be interesting to compare the composition of globulin RNA with that of the whole nuclei and other nuclear RNA fractions as listed in Table I. All the nuclear RNA fractions so far reported, including the globulin, have higher cytidylic and uridylic acid contents than the RNA of whole nuclei. The differences must be due to nuclear RNAs other than those from the nuclear fractions isolated so far. Thus, a complete enumeration of the RNA components present in nuclei must await further fractionation of the total RNA content.

It is clear from the above that the time course of [8-^{14}C]ATP incorporation must be considered as an average over the various components in the globulin fraction, since, after centrifuging the globulin at 40,000 × *g* for 30 min, the pellet has a relatively higher percentage of RNA than the supernatant. Furthermore, both fractions incorporate [^{14}C]ATP (Fig. 5). The pellet material incorporates [^{14}C]ATP for a longer time than the supernatant material. The pellet, thus, not only contains more RNA in proportion to protein, but, also is responsible, at least, for the major features of the time course curve shown in Fig. 3. Fig. 5 also demonstrates a qualitative difference between the supernatant and the pellet, since, in this experiment, the con-

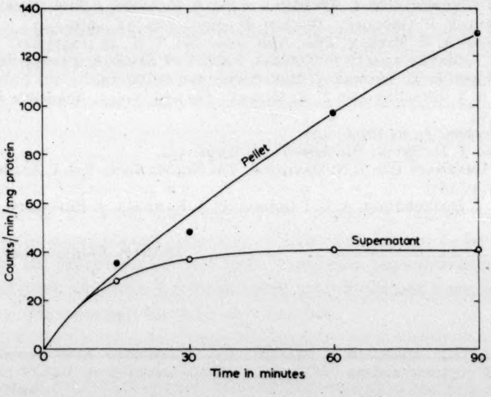

Fig. 5. Conditions of the system were the same as described in Fig. 3 except that the pellet material contained 8.7 mg protein and the supernatant contained 16.7 mg protein; both pellet and supernatant contained approx. 1.4 mg RNA.

centration of RNA was maintained about equal in both fractions. Although the supernatant contained more protein than the pellet, the specific activity of the pellet is much higher than that of the supernatant.

The complexity of the process of [^{14}C]ATP uptake can also be seen from the enhanced incorporation in the presence of a CTP or ATP-generating system (Table II). HECHT *et al.*[8] have shown that AMP can be added proximally to the RNA chain provided the terminal CTP has an unsubstituted 3′ (or adjacent 2′)-hydroxyl group. The heterogeneity of the globulin fraction may indicate that some RNA chains have such a terminus, while other RNA chains may accept the sequential addition of CTP followed by ATP. On the other hand, the incorporation of ATP could also mean two different mechanisms, one of which is CTP-dependent.

It is not known whether the globulin equates or constitutes part of the "pH 5 enzyme" found by HOPKINS *et al.* from isolated thymus nuclei. Should this be the case, the globulin, with its RNA, may, like the "pH 5 enzyme", play a role in nuclear protein synthesis. More information is needed to evaluate the biochemical role of the globulin in isolated nuclei.

ACKNOWLEDGEMENT

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EXTRACELLULAR RIBONUCLEASES FROM *BACILLUS SUBTILIS*[*]

I. CRYSTALLIZATION AND SPECIFICITY

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SUMMARY

1. A method is described for large scale purification of two extracellular RNases obtained from *Bacillus subtilis*. They are here named RNase peak I and RNase peak II respectively.

2. RNase peak II was crystallised in the form of needles from homogeneous preparations obtained by the use of chromatography.

3. The specificity of the RNase peak I and peak II enzymes was quite different from that of bovine pancreatic RNase. They hydrolyzed the "core" remaining after treatment of the yeast RNA fraction with bovine pancreatic RNase. But they hydrolyzed only the secondary phosphate esters of the purine riboside 3′-phosphates in RNA and cyclic guanylic and adenylic acids were then isolated as intermediary products from the dialysate. The mononucleotides and end groups of the oligonucleotides produced by the enzymes were guanylic and adenylic acids.

4. The effects of metallic salts, polyvinyl sulfate and anti-RNase on the activity of RNase peak I and RNase peak II and bovine pancreatic RNase are described.

5. A protein, which has no RNase activity, was obtained in crystalline form by crystallisation of the fraction of peak I and the sedimentation coefficient of this inactive protein was about the same as that of active RNase peak II.

6. A latent RNase, which was activated by 4 *M* urea and was almost completely inhibited by anti-RNases, was detected in the bacterial cells.

INTRODUCTION

Bovine pancreatic RNase was first crystallized by KUNITZ[1] and since then RNases have been isolated from various mammalian organs[2-4], plants[5-8] and microorganisms[9,10]. With the exception of pancreatic RNase and RNase T_1 from takadiastase[11], the RNases so far reported have not been examined physiochemically, because the amount of enzymes available has been small and it has not been possible to obtain them in a state pure enough for such experiments.

Abbreviations: RNA, ribonucleic acid; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuricbenzoate; CM-, carboxymethyl; BSER, *B. subtilisis* extracellular RNase.

[*] A preliminary note on this subject has already been published in the Proceedings of the Eighth Symposium on Nucleic Acid, Research Association of Nucleic Acid, Kyoto, 1959, p. 3.

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NISHIMURA AND NOMURA[12,13] reported that *B. subtilis* produces, as extracellular enzymes, a large quantity of RNases, that one of these enzymes, *i.e.* RNase peak II, could be highly purified by employing the carboxylic acid cation exchange resin IRC-50 (XE-64), and that the specific activity of this purified RNase peak II was comparable to that of crystalline bovine pancreatic RNase.

The present paper describes an improved method for large scale purification and crystallization of RNase peak II, and also the results of a study of the properties of both RNase peaks I and II, particularly their specificity and their immunological and physicochemical properties. The mode of action of these enzymes on RNA appears to be quite different from that of bovine pancreatic RNase. They produced, as terminal nucleotides, only guanylic and adenylic acids.

MATERIALS AND METHODS

The strain of *B. subtilis* used, the culture medium*, the assay procedure, activity unit and specific activity of the enzymes and substrate for RNases were described in previous reports[12,13]. The "core" produced by bovine pancreatic RNase was prepared by the method of TANAKA[14]. The protein content was assayed by the method of LOWRY *et al.*[15], and is expressed as tyrosine equivalent by using a standard tyrosine solution.

The crystalline bovine pancreatic RNase used was a commercial preparation supplied by the Sigma Chemical Company (Lot No. 38–087). Phosphomonoesterase was prepared from hypertrophic human prostate gland as described by MARKHAM AND SMITH[16]. The preparation had no RNase activity and therefore could not have affected the results of the end group determinations.

IRC-50 (XE-64) resin was obtained from the Rohm and Haas Co. and was prepared for use in batches and in columns by the procedure of HIRS[17].

Paper chromatographic solvents

For the separation of cyclic mononucleotides and nucleosides, Solvent I: isopropanol–water (70:30, v/v) with the addition of 0.35 ml of NH_3 solution (0.88 sp. g) for each liter of gas space in the tank, was used[18]. Solvent II: methanol–ethanol–water–conc. HCl (50:25:19:6, v/v/v/v) was used to separate mononucleotides from the enzymic hydrolysate of RNA[19]. For the purpose of identification of guanylic acid isomers Solvent III: satd.$(NH_4)_2SO_4$–isopropanol–1 *M* sodium acetate (80:2:20, v/v/v) was used[16,20]. In all cases ascending chromatography was carried out, using Toyoroshi No. 51 or No. 51 A paper.

Preparation of anti-serum against the RNases of B. subtilis *(anti-BSER)*

Two rabbits were each injected intravenously with 1 ml of crude enzyme preparation fractionated with $(NH_4)_2SO_4$ (Table I, step 5, 1000 units/ml). A total of six injections was given, one injection being given every other day. Because the formation of antibody could not be detected by a precipitin test or by the inhibition of RNase activity, the Freund technique was applied. After homogenizing the enzyme fraction with Freund's adjuvant, about 20,000 enzyme units in a volume of 4.0 ml (Table I, step 6) were injected in four equal portions during 3 months into the subscapular region of each of two rabbits. The rabbits were bled 14 days after the last injection and their sera were separated. The same procedure was used for the preparation of antibody against bovine pancreatic RNase.

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* RNase activity in the culture medium at the maximum stage of production (about 70 h incubation) varied from 5 to 20 units/ml. The cause of this variation is unknown. Overheating of the medium during sterilization must be avoided. Sterilization of the medium was generally carried out at 110° for 15 min in an autoclave. The addition of trace elements (Fe^{++}, Mn^{++}, Mo^{++}, Cu^{++}, Zn^{++}, $B_4O_7^{--}$) reduced the production of both RNases. During cell culture strong aeration was necessary for RNase formation. 150 ml of culture medium was added to a flask (600 ml) and shaken at about 130 rev./min at 30° on a reciprocal shaker with a moving distance of 6 cm.

RESULTS

Large scale purification of the RNase

In order to treat large volumes of culture medium, the batch method, using IRC-50 (XE-64) resin, was applied instead of the ammonium sulfate fractionation previously described[11, 12].

This procedure had the advantage that polysaccharide-like substances could be easily and completely removed.

The culture medium, including the cells, was immediately acidified to pH 2 with 4 N H_2SO_4. After standing overnight at 4°, the suspension was filtered with the aid of Hyflo Super Cel and the filter cake was washed once with water. The filtrate was adjusted to pH 4.2 with 10 N NaOH and to it was then added 0.02 of the filtrate weight of wet resin which had been previously treated with 0.01 M sodium phosphate buffer, pH 7.3. After the suspension had been again adjusted to pH 4.2, it was quickly filtered by suction. The filter cake thus obtained was dispersed in 0.2 M sodium phosphate buffer, pH 7.3, (0.05 of the volume of the initial culture medium); then 8 % NH_3 solution was slowly added until the pH reached 7.3. The suspension was then filtered and the filter cake was washed with a small volume of the same buffer. The combined filtrates were then subjected to ammonium sulfate fractionation at pH 5.9. If adsorption and elution are not done within 15 min, the yield of the enzyme will be decreased.

Table I shows a typical purification and recovery chart obtained at the various steps of this procedure.

As Fig. 1 shows, it was possible to purify the enzyme, step 4 (Table I), by column chromatography using IRC-50 (XE-64) resin as described previously[12, 13]*. Before the enzyme preparation was placed on a column, it was dialysed for about 6 h, adjusted to pH 3.5 and centrifuged to remove the precipitate which always formed.

Each enzyme fraction (peak I and peak II) was collected, dialysed against a hundred times its volume of deionized water at 4° for 4 h, lyophilized and rechromatographed. As Fig. 2 shows, rechromatography of each enzyme on separate columns yielded, in each instance, an single peak, a result which demonstrates that the multiple bands separated from the crude enzyme preparation by the columns of XE-64 could not be attributed to compounds arising from the main constituents by alteration on the column. When rechromatography was done, a load of 5 to 10 mg of enzyme dissolved in 2 to 5 ml of solution was employed, a column of 1.7 cm in diameter being used. The recovery of enzymes by a single passage on the chromatographic column ranged from 75 to 50 %.

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* Dr. T. Tuve pointed out that a CM-cellulose column could also be used for the purification of these bacterial enzymes[21].

TABLE I

PURIFICATION OF RNASE FROM *B. subtilis*

In case of step 1, 2 and 3, protein contents were determined with the dialysed samples.

Steps	*Volume (ml)*	*Total units*	*Total protein content*	*Specific activity (units/μg tyr.)*	*Yield (%)*
1. Culture medium	12,500	72,500	6,580	0.011	100
2. Supernatant after removal of precipitate formed by acidification	12,500	62,500	4,690	0.013	86
3. Eluate from IRC-50 resin (pH, 7.3)	800	33,600	312	0.11	46
4. Precipitate between 0.4 and 0.7 satn. of $(NH_4)_2SO_4$ (310–530 g/l)	50	22,000	125	0.18	30
5. Supernatant at pH 2	50	23,500	111	0.21	32
6. Precipitate between 0.48 and 0.62 satn. of $(NH_4)_2SO_4$ (370–470 g/l)	4	7,300	4.6	1.6	10

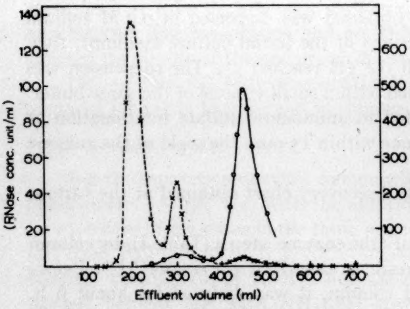

Fig. 1. Column chromatography of RNases from *B. subtilis*. Elution was performed with 0.2 *M* sodium phosphate buffer, pH 5.9 from a column (5 × 20 cm) of Amberlite IRC-50 (XE-64) equilibrated with the same buffer. ———, RNase activity; – – – –, protein content. The flow rate was about 1 ml/cm²/h.

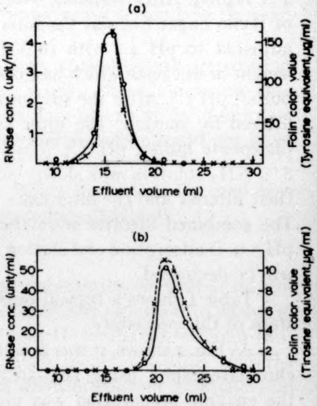

Fig. 2. Rechromatography of (a) RNase peak I and (b) peak II. Experimental conditions were the same as those described for Fig. 1, except that for this procedure a smaller column (0.9 × 20 cm) was used. ———, RNase activity; – – – –, protein content.

Crystallization of RNase peak II

The fraction, peak II, obtained by rechromatography was dialysed for 4 h and lyophilized. It was allowed to contain a small amount of residual sodium phosphate. The resulting wet paste was dissolved in deionized water at pH 5.9 (about 4 mg tyr. equivalent/ml). Saturated ammonium sulfate was then slowly added while the preparation was being stirred, until a very faint turbidity appeared. The suspension was then left at 4° for 2 days. Needle-shaped crystals of RNase gradually formed and during the stirring typical "silkiness" was seen. When, during the next 2 days, saturated $(NH_4)_2SO_4$ was slowly added to about 0.7 saturation, two-thirds of the original protein crystallized out as needles (see Fig. 3). The specific activity (RNase unit/tyr. μg) of crystalline RNase and that of the RNase remaining in the mother liquid were almost the same (almost equal to that of crystalline bovine pancreatic RNase), a result which suggests that the original chromatographically homogeneous enzyme preparation was pure RNase. It was necessary to remove the red-brown colored material, and this was done by elution with the fast running protein peak near the solvent front in order to obtain crystals. Hence a small amount of this material was not completely removed from peak I and peak II by the initial column chromatography and rechromatography was employed to remove this impurity.

Inactive crystalline protein

Crystalline protein which did not possess RNase activity was obtained from the fraction of peak I by the procedure used for the crystallization of RNase peak II. The crystals of this inactive protein appeared in an ammonium sulfate solution more dilute than that required to produce crystals of RNase peak II. When about half of the protein was precipitated as needles (see Fig. 4), all the RNase activity in peak I remained in the mother liquid. The diffusion coefficient of the inactive protein measured in 0.2 *M* sodium phosphate buffer, pH 5.9, at 4°, was $85 \cdot 10^{-8}$ cm²/sec. This value was the same as that of bovine pancreatic RNase measured under the same conditions ($85 \cdot 10^{-8}$ cm²/sec). The sedimentation coefficient of this material was, as will be described below, almost the same as that of active RNase peak II.

Ultracentrifuge analysis

The ultracentrifuge studies were carried out with a Spinco Model E analytical ultracentrifuge. Analyses were performed in 0.2 *M* sodium phosphate buffer, pH 5.9.

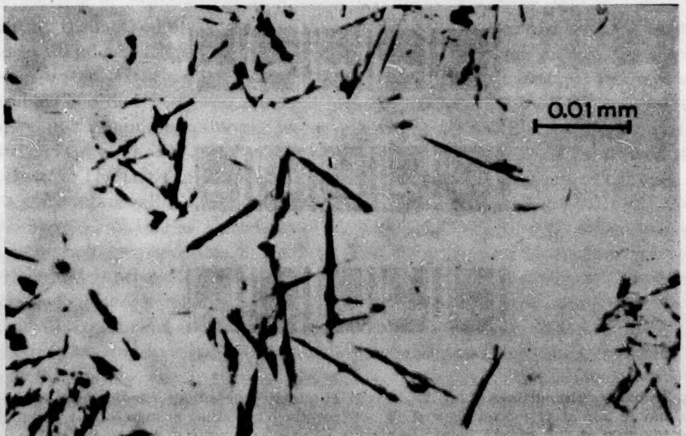

Fig. 3. Crystals of RNase, peak II.

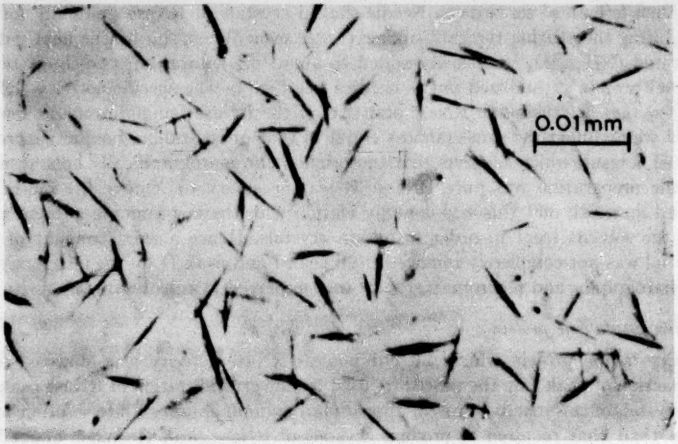

Fig. 4. Crystals of inactive protein from the fraction of peak I.

Fig. 5 shows typical ultracentrifuge pictures with a synthetic boundary cell. Sedimentation coefficients of each sample were determined; (s_{20} was 1.4 for RNase peak II (the rechromatographed preparation), 1.2 for inactive crystalline protein and 1.6 for bovine pancreatic RNase. These data showed that RNase peak II was ultracentrifugically homogeneous and the difference in s_{20} between RNase peak II and pancreatic RNase could scarcely be observed.

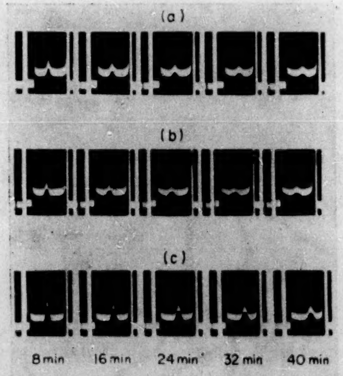

Fig. 5. Sedimentation patterns of (a) RNase peak II (about 3 mg/ml) after sedimentation of 8, 16, 24, 32 and 40 min at 57,000 rev./min at 19°, (b) inactive crystalline protein (about 3 mg/ml) at 57,000 rev./min at 17° and (c) bovine pancreatic RNase (5 mg/ml) at 57,100 rev./min at 28°; solvent 0.2 M sodium phosphate buffer, pH 5.9; bar angle 50°.

The specificities of the enzymes

The following experiments indicated that the specificities of RNase peak I and peak II were quite different from that of bovine pancreatic RNase. The RNase hydrolyzed only secondary phosphate esters of purine riboside 3′-phosphates. No difference has so far been found between the enzyme specificity of RNase peak I and that of peak II.

TABLE II

HYDROLYSIS OF THE "CORE" BY RNASE PEAK I AND PEAK II

The incubation mixtures contained 5 mg of the "core" and indicated amounts of RNase in 0.5 ml 0.067 *M* phosphate buffer, pH 7.3. After 15 min incubation at 30°, the reaction was stopped by adding 0.2 ml of 2 *M* HCl. The precipitate formed was removed by centrifugation and 0.1 ml of the supernatant was added to 10 ml of water. The absorbance at 260 mμ was measured in a Beckman-type spectrophotometer. Complete solubilization of the "core" would give an increase of 0.7 O.D. units.

RNase	*Units of added RNase*	*Absorbance at 260 mμ*	*Increase of absorbance*
Untreated	—	0.47	—
RNase peak II	0.025	0.74	0.27
RNase peak II	0.1	0.73	0.26
RNase peak I	0.025	0.81	0.34
RNase peak I	0.1	0.76	0.30
Bovine pancreatic RNase	0.025	0.45	—0.02
Bovine pancreatic RNase	0.1	0.45	—0.02

Hydrolysis of the "core": The yeast RNA fraction resistant to bovine pancreatic RNase (the "core") was incubated with pancreatic RNase, RNase peak I and peak II respectively. As Table II shows, an increase in the acid soluble component was produced only by RNase peak I and peak II.

Formation of cyclic purine mononucleotides as intermediary products: This was studied by the method of MARKHAM AND SMITH[18]. About 5 units of each RNase were added to 1.0 ml 5 % RNA solution at pH 7.5 in cellulose dialysis tubing suspended in 30 ml of water. After 4.5 h of incubation at 30°, 0.4 of the original RNA became dialysable. The dialysate was collected and lyophilized. Lyophilized samples were dissolved in a minimum volume of water and were then spotted on a filter paper for chromatography. The chromatogram was developed with Solvent I for 16 h at room temperature. The fast running spot (which corresponds to band 6 of MARKHAM AND SMITH and contains all the cyclic nucleotides except that of guanine) was eluted, kept overnight in 0.1 *N* HCl at 4° to split cyclic phosphates to 2′- or 3′-phosphates, and then rechromatographed with Solvent II. The R_F value and absorption spectrum, then showed that, when RNase peak I and peak II were used, only adenylic acid could be detected, although cytidylic and uridylic acids appeared from the spot formed by the control experiment in which pancreatic RNase was used. When, however, the more slowly moving spot (which corresponds to band 5 of MARKHAM AND SMITH) was judged by the R_F value, typical absorption spectrum and fluorescence by u.v. irradiation after treatment with HCl gas, cyclic guanylic acid was detected. The ratio of amounts of cyclic adenylic to cyclic guanylic acids was about 1:6.

Liberation of 3′-guanylic acid from cyclic guanylic acid: Cyclic guanylic acid was incubated with RNase peak II. The chromatogram was developed with Solvent III for 16 h at room temperature. Primarily 3′-guanylic acid was detected in the hydrolysate.

The determination of end groups in RNA hydrolysate: Mononucleotides and terminal groups of oligonucleotides produced by RNase peak II were identified by the method of Markham and Smith[22]. 36 mg of yeast RNA was incubated at 30° for 1 h with 100 units RNase peak II in 1.0 ml 0.025 *M* phosphate buffer, pH 7.5. After this exhaustive digestion, the hydrolysate was kept overnight in 0.1 *M* HCl at 4° and then incubated with prostate phosphomonoesterase at 30°, pH 5.3, for 1 h. To make sure that enough of the enzyme was added to liberate completely the terminal phosphoryl groups, the rate of dephosphorylation of 2′- and 3′-guanylic acids was estimated and 10 times the amount of enzyme required for complete digestion was used. 10 *M* KOH were then added to give a final concentration of 1 *M* and the digest was incubated at 37° for 20 h, so that all the nucleoside derived from the dephosphorylated terminal groups was liberated. KOH in the digest was neutralized by the addition of 60% $HClO_4$. After excess $HClO_4$ had been neutralized by NH_3 solution, the precipitate was removed by centrifugation. The nucleosides were then isolated from the supernatant by chromatographing them with Solvent I as shown in Fig. 6. They were eluted with water and estimated spectrophotometrically; 6.8 µmoles guanosine, 1.9 µmoles adenosine and about 0.4 µmoles of the mixture of cytidine and uridine were produced from 36 mg yeast RNA which consisted of 20.6 µmoles guanylic acid, 19.8 µmoles adenylic acid, 14.0 µmoles cytidylic acid and 19.7 µmoles uridylic acid. Intact RNA treated in the same manner in the absence of RNase gave only a small amount of nucleosides (see Fig. 6). In the same procedure, the formation of end groups in the RNA by the action of RNase peak II was plotted as a function of time as shown in Fig. 7.

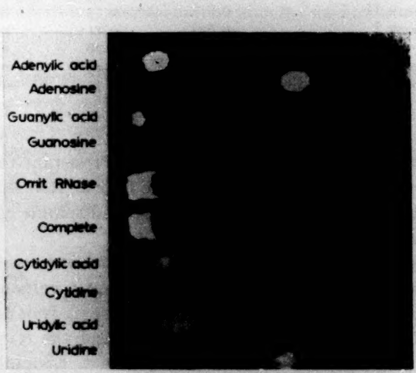

Fig. 6. Demonstration of the presence of end groups in RNA hydrolysate by phosphomonoesterase treatment.

To confirm further whether or not the hydrolysis of secondary phosphate esters of guanosine and adenosine riboside 3′-phosphate was catalysed by a single enzyme, the ratio of guanylic to adenylic acid liberated by the RNase in various purification stages was measured. It was found that the enzyme in the supernatant fraction after the addition of H_2SO_4 and that in the eluate from the XE-64 resin obtained by the batch method both showed the same specificity. 100 units/ml of RNase peak II were respectively heated to 100° for 10 min at pH 4.0 in 0.025 *M* acetate buffer; pH 6.0, in phosphate buffer; and at pH 8.3, in tris(hydroxymethyl)aminomethane buffer. The activity was lost, by about 45 % in the first of these buffers, by 48 % in the second and by 38 % in the third. This partially inactivated enzyme also had the same specificity. Identification of nucleotides was made by ascending chromatography using Solvent II. Zone electrophoresis in starch for 5.5 h at 7 V/cm in tris(hydroxymethyl)-aminomethane buffer, pH 8.3, was carried out. It was found that the enzyme then moved towards the cathode as a single peak. The activity shown as a broad band by electrophoresis in acetate buffer, pH 4.0 migrated towards the cathode. Enzyme aliquots from the fast and slow moving portions of the broad band had the same specificity.

TABLE III

EFFECT OF METALLIC SALTS AND OTHER SUBSTANCES ON THE ACTIVITY OF RNASE

The substance to be tested was dissolved in 0.067 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.3, in case where a metallic salt was added to incubation mixture.

		Percent of activity		
Inhibitor	*Concentration*	*Bovine pancreatic RNase*	*RNase peak I*	*RNase peak II*
PCMB	$1.0 \cdot 10^{-2}$ *M*	100	89	100
Pb	$1.0 \cdot 10^{-2}$ *M*	45	70	91
Mg	$1.0 \cdot 10^{-2}$ *M*	97	73	91
Fe^{++}	$1.0 \cdot 10^{-2}$ *M*	35	43	77
Fe^{+++}	$1.0 \cdot 10^{-2}$ *M*	32	60	65
Ca	$1.0 \cdot 10^{-2}$ *M*	87	—	85
Mn	$1.0 \cdot 10^{-2}$ *M*	84	69	85
Zn	$1.0 \cdot 10^{-2}$ *M*	4.8	35	65
Cu^{++}	$1.0 \cdot 10^{-2}$ *M*	19	59	71
Co	$1.0 \cdot 10^{-2}$ *M*	71	—	61
Hg	$1.0 \cdot 10^{-2}$ *M*	19	15	14
EDTA	$1.7 \cdot 10^{-2}$ *M*	—	—	100
EDTA	$1.7 \cdot 10^{-3}$ *M*	—	100	100
Polyvinyl sulfate	500 μg/ml	28	103	114

The effects of metallic salts and other substances on the activity of the three RNases

The effects of metals on RNase peak I and peak II were almost the same as the effects of metals on bovine pancreatic RNase, although, as Table III shows, Zn^{++} and Cu^{++} had lower inhibitory effects on RNase peak I and peak II than on pancreatic RNase. A notable difference was, however, observed between the effects of polyvinyl sulfate on the bacterial and pancreatic RNases. Although bovine pancreatic RNase was, as other workers[23–25] have also shown, almost completely inhibited by polyvinyl sulfate, RNase peak I and peak II were not inhibited at all at the same concentrations of this substance.

*Anti-*B.subtilis *extracellular RNase*

As Fig. 8 shows, anti-BSER and anti-bovine pancreatic RNase inhibited the enzyme activity of their own antigen at a very low concentration. Anti-BSER did not inhibit pancreatic RNase activity at all. A precipitation reaction between the antiserum and pancreatic RNase could not be detected. The reaction between anti-pancreatic RNase and RNases from *B. subtilis* was also negative. The addition of inactive crystalline protein had no effect on the reaction of anti-BSER with RNase peak II. This was shown by incubation of 0.01 ml antiserum with 10 μg tyr. equivalent, inactive crystalline protein for 20 min before the addition of 0.01 units of RNase peak II.

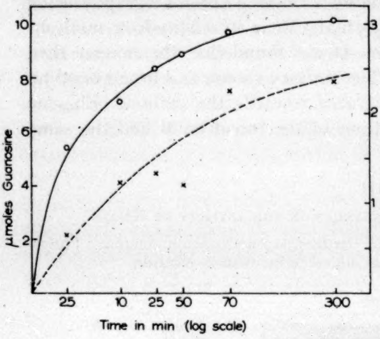

Fig. 7. Formation of end groups during the digestion of RNA by RNase peak II as a function of time. The reaction mixture contained 80 mg yeast RNA (consisting of 45 µmoles guanylic acid, 44 µmoles adenylic acid, 31 µmoles cytidylic acid and 43 µmoles uridylic acid) and 10 units RNase peak II in 1.0 ml 0.033 *M* phosphate buffer, pH 7.3. O—O, guanosine; ×—×, adenosine.

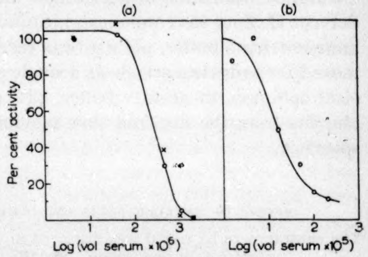

Fig. 8. Neutralization curve of RNase with anti-RNase (a) the reaction of anti-BSER (from rabbit 890) with ×—×, RNase peak I; and O—O, RNase peak II. (b) The reaction of anti-pancreatic RNase (from rabbit 888) with O—O, pancreatic RNase. 0.02 units of each RNase was incubated with antiserum as indicated, in 0.25 ml 0.067 *M* phosphate buffer, pH 7.3 at 4° for 10 min, and 0.25 ml 1% RNA in phosphate buffer was added for the assay of enzyme activity. It was previously confirmed that enzyme-antibody reaction was completed within 5 min.

Activation of latent RNase by urea treatment

NISHIMURA AND MARUO[26] have reported the presence in *B. subtilis* of an intracellular RNase which had properties quite different from those of the extracellular RNases and was completely inhibited by the addition of EDTA. They also found that the cell lysate showed a low degree of alkaline RNase activity, which was inhibited by the addition of anti-BSER. When, however, the lysate was treated with 4 *M* urea, the alkaline RNase activity was increased about 3 times and also, as Table IV shows, the RNase thus activated was completely inhibited by the antiserum. These RNase activities were retained in the supernatant fraction after ultracentrifugation at 100,000 × *g* for 2 h. By this treatment about half of the RNA in the original supernatant was precipitated. Activation by urea of purified RNase peak I and peak II and intracellular acid RNase did not occur.

DISCUSSION

Extracellular RNase peak II from *B. subtilis* is a crystalline RNase and is the first RNase to be crystallized from a source other than mammalian pancreas. Its high specific activity, chromatographic pattern and sedimentation analysis strongly suggests that it is a homogeneous protein.

TABLE IV

ACTIVATION OF LATENT RNase BY UREA TREATMENT

20 g wet cells which were harvested after 70 h cultivation and washed once with water and twice with 0.01 *M* $MgCl_2$ were ground with 60 g of powdered quartz for about 20 min, suspended in 100 ml 0.01 *M* $MgCl_2$ and centrifuged for 40 min at 10,000 × *g*. The supernatant solution was mixed with an equal volume of 8 *M* urea (dissolved in 0.1 *M* phosphate buffer, at pH 6.0 containing 0.02 *M* NaCl) for 2 h at 4°. The untreated sample was only mixed with the above buffer. After the treatment, samples were diluted 5 times with 0.067 *M* pH 7.3 phosphate buffer, for the assay of alkalline RNase, and 10 times with 0.067 *M* pH 5.9 phosphate buffer, for the assay of acid RNase. Incubations with RNA were made for 4 h and 2 h respectively.

Treatment	*RNase activity (increase of absorbance at 260 mμ)*	
	Activity at pH 7.3 in the presence of $0.7 \cdot 10^{-4}$ M EDTA (Alkaline RNase)	*Activity at pH 5.9 (Acid RNase)*
Untreated	0.043	0.223
Untreated + $1.7 \cdot 10^{-2}$ *M* EDTA	—	0.023
Untreated + antiserum	0.000	—
Treated with 4 *M* urea	0.143	0.217
Treated + $1.7 \cdot 10^{-2}$ *M* EDTA	—	0.014
Treated + antiserum	0.020	—

The enzymes, *i.e.*, peak I and peak II, were stable and soluble under acidic conditions. The optimum pH for the action of both RNases was 7.5. The chromatographic behavior of the enzymes was very similar to that of bovine pancreatic RNase although the bacterial enzymes were slightly more acidic than pancreatic RNase. Further, the various metals used had the same effect on both the bacterial and pancreatic enzymes. The sedimentation coefficient of RNase peak II was about the same as that of pancreatic RNase. It was extremely interesting to find that, although the enzymes RNase peak I and II were quite similar in respect of the properties just mentioned, they were, when they were compared with pancreatic RNase, quite different in their specificity. Preliminary evidence[27] suggested that the amino acid composition of RNase peak II was different from that of bovine pancreatic RNase. Studies of comparative biochemistry and relation between enzymic activity and protein structure will be helped by the isolation of this new RNase.

SATO-ASANO[28] has shown that RNase T_1 in takadiastase obtained from *Aspergillus oryzae* by EGAMI and his collaborators specifically hydrolyzes secondary phosphate esters of purine riboside 3′-phosphate. Her experiments showed, however, that this action of RNase T_1 formed only guanylic acid as the digestion product of RNA; it did not form adenylic acid. RNase T_1, compared with pancreatic RNase, appeared to be a very acidic protein. Anti-BSER did not react with RNase T_1. It could therefore be said that the extracellular RNases from *B. subtilis* were also different in many respects from RNase T_1.

It is possible to consider that RNase peak II consists of two enzymes, one of which liberates guanylic acid from RNA, while the other liberates adenylic acid. Yet the enzyme, at various stages of the purification, and also the partially heat-inactivated enzyme produced guanylic and adenylic acids from RNA at constant rates. Crystalline RNase moreover, had the same specific activity as the enzyme remaining in the mother liquid. In zone electrophoresis in starch, RNase peak II migrated as a single peak. It is therefore felt that a single enzyme is responsible for the liberation of both guanylic and adenylic acids from RNA.

Crystalline protein, which did not possess RNase activity, was obtained from the fraction of peak I. The physicochemical properties of this protein were very similar to those of RNase peak II. The biological interpretation of this fact is at present unknown, but the possibility may be considered that this crystalline protein is a precursor of RNase or a degraded product of it produced by the action of the exoprotease of this bacterium formed during its growth.

Recently DICKMAN *et al.*[29] announced the presence of latent RNase in mouse pancreas, and ELSON[9] reported that ribonucleoprotein in *Escherichia coli* contained latent RNase which was activated by urea treatment. The properties of this enzyme resembled those of pancreatic RNase. As Table IV shows, the experiments here described indicate that latent RNase activated by urea is present in cells of *B. subtilis*. This RNase might be the same as extracellular RNase, because its activity was inhibited by anti-BSER. This enzyme did not attach to the microsomal fraction, but was in contrast with the results obtained by ELSON with *E. coli*, found in the supernatant fraction. ROTH[30] described an RNase inhibitor which was found in the supernatant fraction prepared from rat liver homogenate. It is therefore possible that such an inhibitor is present in the cells of *B. subtilis* and is combined with RNase before the excretion of this enzyme. Investigation of this possibility has not been completed.

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YEAST PEPTIDYL-NUCLEOTIDATES; ISOLATION OF DINUCLEOTIDE DERIVATIVES INCLUDING ALANYL-(3′-[5′-ADENYLYL])-5′-URIDYLATE

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SUMMARY

The isolation of individual peptidyl-nucleotidates from ethanolic and trichloroacetic acid extracts of a 48 h culture of brewers' yeast has been accomplished using electrophoresis and chromatography on paper and ion-exchange resins.

Structural study of the individual compounds revealed that they were nucleotide-5′-phosphoanhydrides. Three of those investigated were derived from the mononucleotide, uridine-5′-phosphate, and five from the dinucleotide, 3′-(5′-adenylyl)-uridine-5′-phosphate. Of the latter group, one proved to be alanyl-(3′-[5′-adenylyl])-5′-uridylate, analogous with the amino acid adenylates participating in protein synthesis. Other members of the group have similar structures in which the α-alanine unit is replaced by peptide residues containing some or all of the amino acid residues of α-alanine, arginine, aspartic acid, cystine, glutamic acid, leucine and valine.

INTRODUCTION

Understanding of the course of the biosynthesis of proteins is hampered by lack of detailed knowledge of the intermediates involved, and, indeed, the necessity for such intermediates apart from the activated amino acids is discounted in some versions of the template theory (see *e.g.* DOUNCE[1]; review by HARRIS[2]). However, the presence of intermediates in the synthesis of ovalbumin was deduced by STEINBERG AND ANFINSEN[3], and a number of investigators[4] have found peptides, regarded as precursors of proteins, to be present in extracts of various microorganisms. Recent developments are the reports or inferences that peptide derivatives of nucleotides are present in extracts from a number of sources, including bakers' yeast[5-7], brewers' yeast[8-10], *Candida utilis*[11], *Azotobacter vinelandii*[12], *Chlorella*[6,7], *Staphylococcus aureus*[13], *Streptococcus faecalis*[14], *Escherichia coli*, *Bacillus subtilis*[15,16] and liver cytoplasm[17].

In most instances no pure compounds of this class have been isolated and comparison of the properties reported for the groups of substances isolated by various workers reveals that considerable differences exist. For instance, some of the substances migrate towards the anode[13,14] on electrophoresis in acidic buffer solutions while others move towards the cathode[5-10,17]. The compounds most likely to be intermediates in protein synthesis are those which possess either amino- or carboxyl-groupings in activated form to facilitate combination into protein. Accordingly, in the case of the substances from brewers' yeast[8-10] and from liver cytoplasm[17] attention has been confined to those which have acylating properties as reflected in their yielding hydroxamic acids on reaction with neutral hydroxylamine[18, 19]. It is remarkable that nearly all these compounds migrate towards the cathode when submitted to electrophoresis at pH 4.0 (see ref. 10). Of such compounds one was isolated earlier and shown by degradation[9, 10] and by synthesis[20] to be arginylalanylarginylalanyl-5'-uridylate (see ref. 21). It was hence closely related chemically to the amino acid-5'-adenylates[22], now generally recognised to be the active form of amino acids participating in protein synthesis in various cells[18, 19, 23, 24] including yeast[25]. It and similar compounds are therefore referred to as peptidyl-nucleotidates as distinct from the less well-defined nucleopeptide preparations.

Of the other nucleotide–peptide complexes, GILBERT AND YEMM[11] proposed that one in *Candida utilis* was derived not from a nucleoside monophosphate, like the compound from brewers' yeast, but from uridine diphosphate. Furthermore, BERNLOHR AND WEBSTER[12] suggested that compounds reactive towards hydroxylamine in extracts of *Azotobacter vinelandii* are derived from poly- rather than mononucleotides and polynucleotide-amino acid compounds have been postulated as intermediates for protein[26-28]. Accordingly, further examination of the acylating components in extracts of a brewing yeast has been undertaken to gain insight into the range of their chemical structures. These compounds are unstable, particularly in alkaline solutions, and any step in working them up or chromatographing them which involves the use of alkaline conditions decomposes them. This may account for the fact that some workers have isolated anode-migratory nucleopeptides and that others have failed to recognize acylating properties in their products. For this reason, it remains to be seen whether or not the peptides observed, for example, in *E. coli*[4] were originally attached to nucleotides.

MATERIALS AND METHODS

Culture and extraction of yeast: This was carried out as described earlier[10] with the exception that extraction with hot aqueous ethanol was omitted.

Ion-exchange resins: These were treated for use as outlined in a previous communication[10].

Analytical methods

The determinations of purines, pyrimidines, ribose, phosphate and amino acids were effected largely as before[10]. It was found convenient, however, to separate the purines and pyrimidines on acid-washed Whatman No. 3 papers.

The peptide moiety of the peptidyl-nucleotidates was removed by treating these compounds with 0.3 *N* potassium hydroxide for 16 h at 37°. The resulting solution was neutralized with perchloric acid at 0° and then kept for 16 h at 0° to precipitate potassium perchlorate. This was filtered off, using acid-washed Whatman No. 42 paper, and thoroughly washed with cold water. The combined filtrate and washings were concentrated at room temperature *in vacuo*, further potassium perchlorate removed in the same way and the procedure repeated until the amount of potassium perchlorate separating was negligible. The concentrate was chromatographed overnight in butanol–acetic acid–water (4:1:1, v/v) on acid-washed Whatman No. 3 paper, the peptide zone located by means of the ninhydrin reagent and eluted by means of water. The extract was concentrated and submitted to electrophoresis on paper[10] to separate nucleotides migrating towards the anode from the peptide, which was then eluted and analyzed for amino acids[10].

The nucleotide portions of the peptidyl-nucleotidates were obtained by treatment of these compounds with neutral hydroxylamine and separation of the products by electrophoresis as before. The nucleotides were eluted and chromatographed in KIRBY's solvent No. II (see ref. 29) and the constituent bases identified by hydrolysis and chromatography[10].

Peptidyl-nucleotidates in solution were estimated by reaction with hydroxylamine and ferric chloride and measurement of the resulting red colour[18,19]. The reaction of peptidyl-nucleotidates with periodate was measured by means of the decrease in the u.v. light absorption of the solution[30].

Isolation of peptidyl-nucleotidates

Cold ethanol extract: The extract was concentrated at room temperature and the residue submitted directly to electrophoresis on Whatman No. 3 paper at pH 4.0. Three components reactive towards hydroxylamine migrated towards the cathode. The major component, of intermediate mobility, was eluted and the extract freed of amino acids and peptide contaminants by passage down a column of Amberlite CG 50. The effluent was loaded on to a column of Dowex-1 (formate) and the peptidyl-nucleotidates eluted in a formic acid gradient (0.05 *M*–4.0 *M*). Several compounds were thereby detected of which the two most reactive towards hydroxylamine were present respectively in the direct effluent and the eluate obtained using 2.0 *M* formic acid (Fig. 1). The former (D_4) behaved as a single compound on chromatography on paper in either of the two solvent systems, butanol–acetic acid–water (4:1:1, v/v) or *n*-propanol–ethyl acetate–water (7:1:2, v/v) containing acetic acid[10]. It was therefore analyzed further (*cf.* Table I).

Cold aqueous ethanol extract: The method used for working up this extract is shown in Scheme I. The peptidyl-nucleotidates desorbed from Dowex-1 (chloride) by acetate buffer largely pass through Dowex-1 (formate) in the presence of excess acetate, but

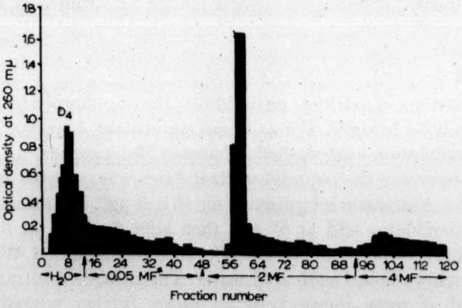


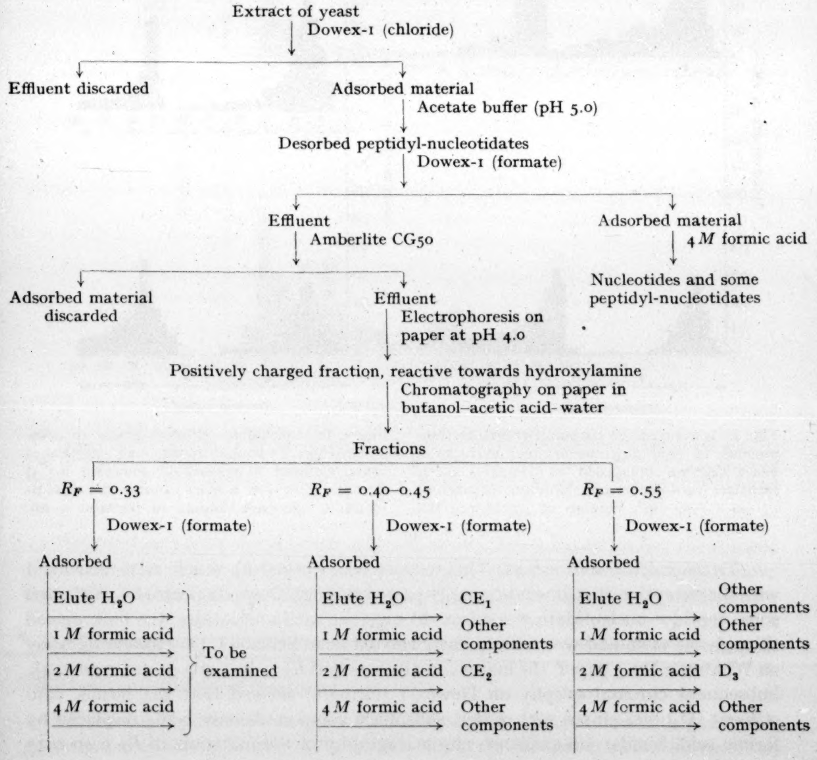
Fig. 1. Ion exchange chromatogram of components in a cold ethanol extract of yeast. Column composed of Dowex-1 (× 4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm². Volume of fraction 5 ml. *F = formic acid.

TABLE I

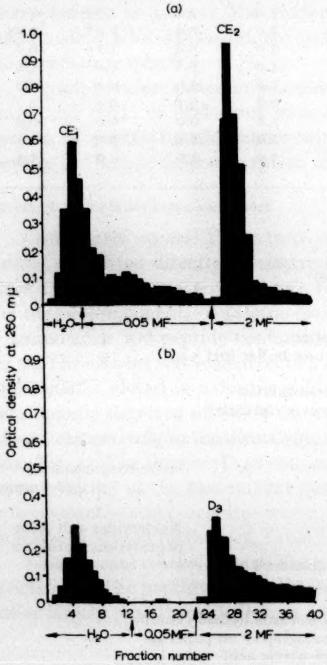
GENERAL ANALYSIS OF PEPTIDYL-NUCLEOTIDATES FROM YEAST

Component analysed	Alcohol-soluble				Soluble only in trichloroacetic acid			
	CE_1	CE_2	D_3	D_4	TCA_1	TCA_2	D_1	D_2
Adenine	1.0	0.0	0.0	0.0	1.0	1.0	1.0	1.2
Uracil	1.0	1.0	0.97	1.12	1.0	1.0	0.9	1.0
Phosphorus	2.0	1.0	1.0	1.0	2.0	2.0	1.75	2.0
Ribose	2.0	1.0	1.1	1.3	2.0	2.0	2.06	2.54
α-alanine	2.2	4.2	5.05	4.0	3.0	5.8	1.15	6.6
Arginine	10.0	7.0	4.1	2.3	14.0	8.8	0.0	5.6
Aspartic acid	0.7	1.1	0.0	0.0	0.0	0.0	0.0	0.0
Cystine	0.7	0.0	1.4	1.4	0.8	3.2	0.0	2.7
Glutamic acid	3.7	1.6	0.0	0.0	1.8	0.0	0.0	0.0
Leucine	1.9	1.26	6.5	1.6	2.0	1.2	0.0	6.3
Valine	1.0	1.0	6.5	2.4	2.0	2.0	0.0	5.3

Scheme I


Extract of yeast
Dowex-1 (chloride)
Effluent discarded
Adsorbed material
Acetate buffer (pH 5.0)
Desorbed peptidyl-nucleotidates
Dowex-1 (formate)
Effluent
Amberlite CG50
Adsorbed material
4 M formic acid
Adsorbed material discarded
Effluent
Electrophoresis on paper at pH 4.0
Nucleotides and some peptidyl-nucleotidates
Positively charged fraction, reactive towards hydroxylamine
Chromatography on paper in butanol–acetic acid–water
Fractions
R_F = 0.33
Dowex-1 (formate)
Adsorbed
Elute H_2O
1 M formic acid
2 M formic acid
4 M formic acid
To be examined
R_F = 0.40–0.45
Dowex-1 (formate)
Adsorbed
Elute H_2O
CE_1
1 M formic acid
Other components
2 M formic acid
CE_2
4 M formic acid
Other components
R_F = 0.55
Dowex-1 (formate)
Adsorbed
Elute H_2O
Other components
1 M formic acid
Other components
2 M formic acid
D_3
4 M formic acid
Other components

after successively treating with Amberlite CG 50 and chromatographing on paper they are more readily adsorbed on the Dowex-1 (formate) (17×2.25 cm^2). Elution first with water and then with formic acid solutions of increasing strengths (0.05 *M*–4.0 *M*) gave a range of peptidyl-nucleotidates of which the most reactive towards hydroxylamine were the components CE$_1$, CE$_2$, and D$_3$, which were accordingly studied further (Fig. 2).



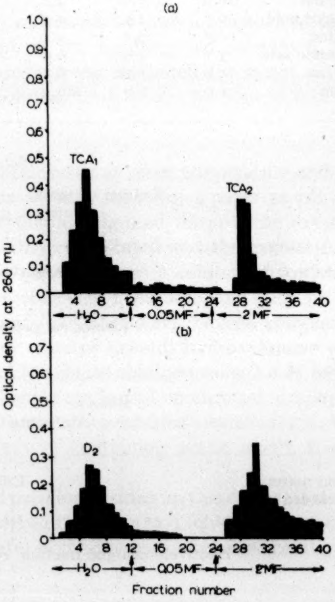


Fig. 2. Ion exchange chromatograms of components in cold aqueous ethanol extracts of yeast. Column composed of Dowex-1 (× 4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm^2. Volume of fraction 5 ml.

Fig. 3. Ion exchange chromatograms of components in cold trichloroacetic acid extracts of yeast. Column composed of Dowex-1 (× 4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm^2. Volume of fraction 5 ml.

Trichloroacetic acid extract: This extract was worked up much as in Scheme I with the exception that it was originally passed through Dowex-1 (formate). Although some peptidyl-nucleotidate was adsorbed together with nucleotides, the bulk passed through the resin and was subsequently treated as in Scheme I. On chromatography on Whatman No. 3 paper, the major constituents had R_F values of 0.55 and 0.40–0.45. Subsequent chromatography on Dowex-1 (formate) showed that the former constituent (D$_2$) was eluted with water, subsidiary compounds only being displaced by formic acid. Similar ion-exchange chromatography of the materials of R_F 0.40–0.45

gave two main components, one (TCA_1) being displaced from the resin by water and the other (TCA_2) being displaced by 2 *M* formic acid (Fig. 3).

In experiments designed to handle larger quantities, chromatography on Whatman No. 3 paper was replaced by chromatography on a cellulose column (91 cm × 12.25 cm²) in butanol–acetic acid–water (4:1:1, v/v). The effluent was collected in 5 ml fractions and the progress of separation followed by means of the u.v. absorption of the fractions at 260 mμ (Fig. 4).

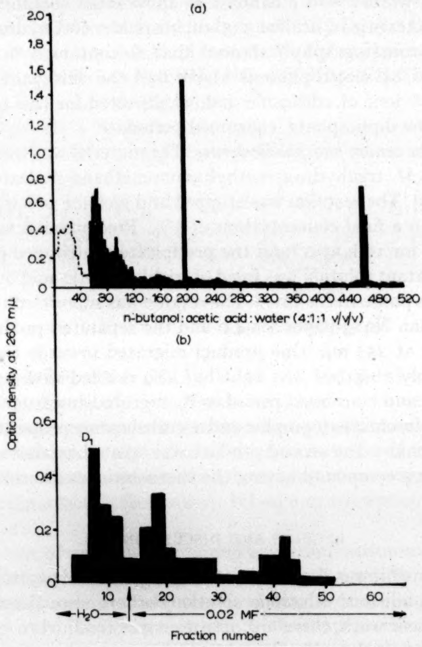


Fig. 4a. Chromatogram of components in cold trichloroacetic acid extract of yeast. Column composed of cellulose. Column dimensions 91 cm × 12.25 cm². Volume of fraction 5 ml.
Fig. 4b. Ion exchange chromatogram of pooled fractions 45–80 from (a). Column composed of Dowex-1 (× 4) formate (200–400 mesh). Column dimensions 17 cm × 1.76 cm². Volume of fraction 5 ml.

Of the four major "peaks" the first eluted was diluted with water, the organic phase removed and the remaining aqueous phase extracted with ether to remove butanol and acetic acid. The aqueous solution was passed through a column of Dowex-1 (formate). The main component (D_1) passed through the column on washing with water. The remaining compounds present were displaced by means of 0.02 *M* formic acid.

Structural examination of compound D_1

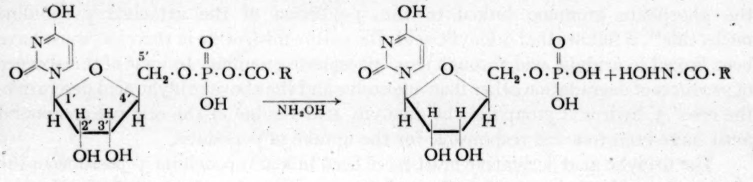
By means of pancreatic ribonuclease: Compound D_1 was treated with pancreatic ribonuclease in acetate buffer solution at pH 5.4. As this pH is well removed from the optimum (7.5)[31] for the enzyme, a relatively large amount of the enzyme was employed. The mixture was kept for 16 h at 37° after which the reaction was terminated and enzyme removed[32]. The solution was concentrated at room temperature and the product submitted to electrophoresis on paper at pH 4.0. Two components were detected by examination of the dried electropherogram in u.v. light at 254 mμ. One migrated towards the anode and reacted with hydroxylamine to form α-alanine hydroxamate and a nucleotide. Chromatography of the nucleotide in the solvent systems isopropanol–water[33] and ethanol–1 M ammonium acetate[33] showed that it had R_F values characteristic of uridine 3′,5′-diphosphate, and hydrolysis with formic acid followed by chromatography[10] showed that it contained uracil. The second component separated by electrophoresis above had the chromatographic and u.v. light absorption behaviour of adenosine and, as expected for this compound and in contrast to the uridine diphosphate, consumed periodate.

By means of snake venom phosphodiesterase: The material was treated with Russell viper venom in 0.05 M tris(hydroxymethyl)aminomethane–maleate buffer solution at pH 7.0 (see ref. 34). The reaction was stopped and enzyme precipitated by adding trichloroacetic acid to a final concentration of 5 %. Precipitation was completed by keeping the mixture for 16 h at 0° and the precipitate centrifuged off at 0° at 5,000 rev./min. The supernatant solution was freed of trichloroacetic acid by extraction with ether and the aqueous phase freeze-dried. The residue was submitted to electrophoresis at pH 4.0 on Whatman No. 3 paper for 4 h and the separated products detected by their u.v. absorption at 254 mμ. One product migrated towards the anode and the second, which not only absorbed u.v. light but also reacted with hydroxylamine to form a hydroxamate and consumed periodate[30], migrated towards the cathode. The first product showed the chromatographic and u.v. absorption properties characteristic of adenosine-5′-phosphate. The second product was treated as above with potassium hydroxide whereupon a compound having the characteristics of uridine-5′-phosphate was formed.

RESULTS AND DISCUSSION

In earlier investigations[10] it was observed that, although some of peptidyl-nucleotidates withstood heating in aqueous ethanolic solution, others were decomposed by this treatment. In the present work, therefore, attention was confined to extracts obtained from yeast using successively cold ethanol, cold aqueous ethanol and cold aqueous trichloroacetic acid. The adsorption on Dowex-1 (chloride) of materials reactive towards hydroxylamine and their selective elution from the resin by means of acetate buffer at pH 5.0 resulted in quantitative recovery of the colour-forming materials[18, 19]. This initial concentration procedure probably, therefore, yields a product representative of the compounds present in the original extracts. On the other hand, some loss of peptidyl-nucleotidate was incurred during the later treatment with Amberlite CG 50 (see MATERIALS AND METHODS) but this step in the purification was usefully retained as it ensured the absence of free amino acids or peptides.

Of the large number of acylating compounds detected in the alcoholic and trichloroacetic acid extracts of the yeast, four in each extract were selected for further study as being present in readily workable quantities. The results of the analysis[10] of these compounds are shown in Table I. It is remarkable that, in common with the first peptidyl-nucleotidate isolated from yeast[9, 10], all the compounds contain uracil, and compounds CE_2, D_3 and D_4 contain only this base of those commonly derived from nucleic acids. In these three compounds the uracil was in the form of a uridine-5′-phosphate residue as this nucleotide was liberated on degradation of the original compounds with alkali[10]. The uracil, phosphorus and ribose (Table I) were thus accounted for. Each of the three compounds reacted with neutral hydroxylamine to give not only the nucleotide but also a peptide hydroxamate which behaved as a single unit on chromatography. Moreover, each consumed periodate, presumably as a result of the reaction of the reagent with the 2′,3′-*cis*-hydroxyl groups of the ribose moiety. It follows, therefore, that the compounds CE_2, D_3 and D_4 have structures (I), analogous with that of arginylalanylarginylalanyl-5′-uridylate (I; $R = CH(CH_3)\cdot NH\cdot CO\cdot CH[(CH_2)_3\cdot NH\cdot C(NH_2){:}NH]\cdot NH\cdot CO(CH_3)\cdot NH\cdot CO\cdot CH[(CH_2)_3\cdot NH\cdot C(NH_2){:}NH]NH_2$) and react with hydroxylamine thus:


OH
N
O
N
O
5′
$CH_2\cdot O\cdot P\cdot O\cdot CO\cdot R$
OH
1′
4′
H
H
H
H
2′
3′
OH OH
NH_2OH
OH
N
O
N
O
$CH_2\cdot O\cdot P\cdot OH + HOHN\cdot CO\cdot R$
OH
H
H
H
H
OH OH

Peptidyl-nucleotidate (I) Uridine-5′-phosphate Peptide hydroxamate

The compounds CE_1, TCA_1, TCA_2, D_1 and D_2 contained adenine in addition to uracil, and on degradation with alkali or neutral hydroxylamine gave not a mixture of the corresponding mononucleotides but a hitherto unknown oligonucleotide. The chromatographic behaviour (R_F-value 0.65–0.68 in solvent II of KIRBY[29]) and molar ratios (Table I) for adenine, uracil, phosphorus and ribose suggested that this oligonucleotide was a dinucleotide (see below). It had u.v. absorption almost identical with that of uridylic acid.

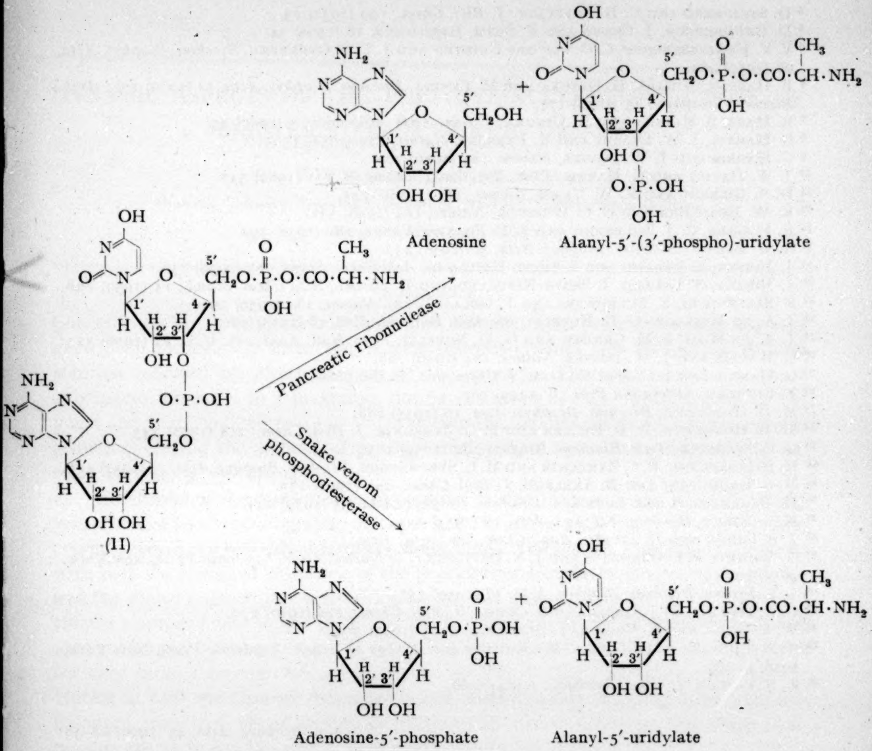
From the point of view of their amino acid composition most of the compounds isolated contained a relatively high proportion of arginine and α-alanine, again resembling arginylalanylarginylalanyl-5′-uridylate and recalling to mind the composition of nucleoprotein. In this connection it is perhaps a curious feature of the whole peptidyl-nucleotidate concentrate from the particular yeast used, that only the amino acids listed in Table I occur to any considerable extent. Comparison of this amino acid pattern with the amino acid composition of whole brewers' yeast (*cf.* EDDY[35]) reveals considerable differences although it is nevertheless true to say that, of the combined amino acids in yeast, α-alanine, arginine, leucine and valine are present in largest amounts. Further, TAYLOR[36] has observed that a 40-h culture of yeast contains a high proportion of extractable arginine but relatively low proportions of tyrosine and histidine.

Compound D_1 was of particular interest because it contained only one amino acid, namely α-alanine. As, moreover, it gave the above mentioned oligonucleotide on degradation, it afforded a particularly suitable subject for structural analysis especially in view of its bearing also on the structures of all the compounds CE_1, TCA_1, TCA_2 and D_2. Degradation with alkali gave, in addition to the oligonucleotide, a compound which co-chromatographed with α-alanine while treatment with hydroxylamine afforded a substance having the chromatographic behaviour of N-[2-amino-2-methylacetyl] hydroxylamine. On the basis of this dual chromatographic evidence it is concluded that α-alanine is present as a single amino acid unit in the original compound. In view of the molar ratios between the constituents of compound D_1 (Table I) the oligonucleotide formed on degradation must be a dinucleotide as suggested above.

That one at least of the mononucleotide moieties of the dinucleotide probably bore only a 5′-substituent was shown by the uptake of periodate by the original compound. This mononucleotide was shown to be adenylic acid by hydrolysis of the parent compound by pancreatic ribonuclease at pH 5.4, whereupon adenosine, which reacts with periodate, and a derivative of uridylic acid, which failed so to react, was formed. Under the conditions used the uridylic acid derivative retained the acylating properties of the original molecule. As pancreatic ribonuclease specifically hydrolyzes phosphate linkages at position 5′ of purine nucleotides of, for example, ribonucleic acid, leaving the phosphate grouping linked to the 3′-position of the attached pyrimidine nucleotide[31], it follows that adenylic acid, the purine nucleotide in this case, must have been linked to uridylic acid through its 5′-phosphate grouping. In view of the absence of products of degradation other than adenosine and the above uridylic acid derivative, the *cis*-2′,3′-hydroxyl groups of the adenylic acid residue of the original compound must have been free and responsible for the uptake of periodate.

The uridylic acid derivative must have been linked through its 3′-position to the 5′-adenylic acid residue as the ribonuclease is specific for the hydrolysis of 5′,3′-internucleotide bonds[31]. The lack of reactivity of this part of the molecule towards periodate was thus explained and only positions 2′- and 5′- of the uridylic acid moiety were left as alternative points of attachment of the second phosphate grouping of the dinucleotide. Of these alternatives, the 5′-position was the one in question as degradation of the uridylic acid derivative by means of alkali gave uridine-3′,5′-diphosphate with concomitant liberation of α-alanine. This latter amino acid must have been bound through a phosphoanhydride linkage therefore to either the 3′- or the 5′-position of the uridine diphosphate. By analogy with the peptidyl-nucleotidates above, containing only uridylic acid residues, the latter alternative appears most likely. Furthermore, it is improbable that ribonuclease, a phosphodiesterase, would attack a trisubstituted phosphate grouping as would be necessary were the amino acid bound to the internucleotide phosphate grouping. Confirmation of these ideas was obtained by hydrolyzing compound D_1 with a ribonuclease of different specificity from the pancreatic enzyme, namely the phosphodiesterase from Russell viper venom. This enzyme hydrolyzes, for example, ribonucleic acid to liberate the 5′-nucleotides of both purines and pyrimidines[31]. In conformity, compound D_1 gave rise to 5′-adenylic acid and a uridylic acid derivative which in this case consumed periodate. As this latter derivative still contained the reactive amino acid-phosphoanhydride grouping, it follows that this was in the 5′-position. Consequently, compound D_1 has the structure (II), *i.e.*, alanyl-(3′-[5′-adenylyl])-5′uridylate, which, to our knowledge, is the first activated nucleotide-derivative of a single amino acid isolated from the cell itself as against products of *in vitro* enzymic reaction.

It seems probable by analogy that compounds CE_1, TCA_1, TCA_2 and D_2 are corresponding peptidyl-(3′-[5′-adenylyl])-5′-uridylates and in this event all the compounds from yeast so far examined are anhydrides of either peptides or an amino acid with uridine-5′-phosphate or with the above derived dinucleotide. The dinucleotide


(II)
Pancreatic ribonuclease
Snake venom phosphodiesterase
Adenosine
Alanyl-5′-(3′-phospho)-uridylate
Adenosine-5′-phosphate
Alanyl-5′-uridylate

derivatives are particularly interesting in that they provide a chemical bridge between, on the one hand, amino acid adenylates and simple peptide-nucleotidates and, on the other hand, the S-ribonucleic acid–amino acid complexes, which have been proposed to play a part in protein synthesis in various cells[18–26]. Moreover, they are the first isolated representatives of the polynucleotide–amino acid complexes earlier postulated to exist in cell extracts[12, 27, 28] (see INTRODUCTION).

ACKNOWLEDGEMENTS

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DYNAMIC ASPECTS OF THE PEPTIDYL-NUCLEOTIDATE POOL OF BREWER'S YEAST DURING GROWTH

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SUMMARY

Concentrates of peptidyl-nucleotidates were obtained from yeast at various stages of growth by successively extracting it with aqueous ethanol and trichloroacetic acid solution followed by electrophoresis of the extracts. The total amount of these substances increased to a maximum during the onset of logarithmic growth of the yeast and then declined to a low value during the logarithmic phase. As the rate of growth decreased the amount of peptidyl-nucleotidate again increased, only to fall once more as the culture aged.

Resolution of the concentrates of peptidyl-nucleotidates at each stage of growth was achieved by chromatography on paper and the amino acid, purine and pyrimidine composition of the individual fractions determined. The results, taken in conjunction with both the chemical structures of the peptidyl-nucleotidates as already elucidated and the above data on the derived hydroxamates, suggest that the peptidyl-nucleotidates examined participate in the synthesis of nucleoprotein. It appears that they are synthesized by the yeast cell in excess during early growth but are then organized for very rapid turnover during logarithmic growth so that their stationary concentration at any one time at this stage is low. Subsequently, as growth becomes unbalanced the synthesis of certain peptidyl-nucleotidates continues but these compounds are no longer integrated with others and hence accumulate.

INTRODUCTION

In the previous paper[1] it was shown that peptidyl-nucleotidates from yeast are 5'-phosphoanhydrides derived from a mononucleotide or an oligonucleotide on the one hand and a peptide or amino acid on the other. They were derived from a 48 h culture of yeast and it was of interest further to discover whether or not they were present in the cells at earlier stages of growth and how the pool of peptidyl-nucleotidates varied during the life of the culture. The present communication describes attempts to obtain information on these aspects of yeast cell growth.

MATERIAL AND METHODS

Culture of yeast

Saccharomyces cerevisiae (No. 240, British National Collection of Yeast Cultures) was grown for 48 h in shake culture in MYGP. The yeast was centrifuged off and a

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Abbreviation: MYGP, maltose extract–yeast extract–glucose–peptone medium.

sample removed for analysis as below. The bulk was immediately inoculated into fresh MYGP in which it was grown with shaking and samples removed as required.

Extraction of yeast

The yeast was centrifuged off at intervals and immediately treated with cold ethanol to inactivate it. It was washed quickly with a mixture (1:1, v/v) of ethanol and ether and then with ether and finally placed in a mixture of ether and solid carbon dioxide to disrupt it. The cell residue was then dried *in vacuo* over phosphorus pentoxide and weighed (Table I).

TABLE I

WEIGHT OF YEAST AND VARIOUS EXTRACTS DURING GROWTH

Growth period (h)	*Weight of yeast (mg)*	*Weight of total extracted material (%)*	*Hydroxamate yielded by extract (μmole tyrosine hydroxamate)*					
			(a) per mg yeast × 10³			*(b) per total culture*		
			Alcohol extract	*TCA extract*	*Total*	*Alcohol extract*	*TCA extract*	*Total*
0.0	190	24.3	5.75	0.67	6.42	1.09	0.127	1.21
1.0	225	27.2	8.75	3.08	11.83	1.968	0.693	2.66
2.0	247	27.5	7.30	2.02	9.32	1.803	0.490	2.30
3.0	291	—	3.70	1.07	4.77	1.076	0.311	1.38
5.0	333	30.7	1.62	0.97	2.59	0.539	0.323	0.86
5.6	390	24.5	1.50	1.17	2.67	0.585	0.456	1.04
6.6	404	21.1	2.38	0.53	2.91	0.961	0.214	1.17
23.6	476	24.8	1.70	0.63	2.33	0.809	0.299	1.10
29.6	439	24.4	3.99	0.81	4.80	1.751	0.355	2.10
49.8	439	22.7	2.10	0.75	2.85	0.921	0.329	—

One fraction of the peptidyl-nucleotidates (a) was extracted from the residues by successive elution with 50 % ethanol (3 × 25 ml) for 3 h at room temperature. The extracts were combined and concentrated to dryness at room temperature. The cell debris remaining from the extraction was washed with ether and dried *in vacuo* as above. Similar results were obtained when the ethanol used for killing the original cells was diluted directly to 50 % with water and extraction continued with 50 % ethanol as above. A second fraction of peptidyl-nucleotidates (b) was obtained from the dried debris (100 mg) by mixing it with ice-cold 10 % trichloroacetic acid solution (10 ml), freezing the mixture and keeping it overnight. The mixture was allowed to thaw, the solid rapidly centrifuged off and the supernatant solution immediately extracted with ether to remove the bulk of trichloroacetic acid. The remainder of the acid was removed by continuous extraction with ether for 16 h and the aqueous residue then concentrated to dryness at room temperature.

Concentration of peptidyl-nucleotidates

Fraction a was dissolved in 50 % ethanol (2 ml) and fraction b in water (2 ml), the solutions divided into halves and each half submitted to electrophoresis in acetate buffer at pH 4.0 on Whatman No. 3 paper for 16 h at 10–20 V/cm.

From one of each pair of electropherograms, the material migrating towards the cathode was eluted by extraction with cold water (3 × 15 min) and the total extract in each case concentrated *in vacuo* at room temperature (Concentrates I).

Formation and chromatography of hydroxamates

The second member of each pair of electropherograms was treated with the hydroxylamine reagent[2] and the hydroxamates formed were eluted with water. The extracts were concentrated at room temperature to 2 ml. A sample (1 ml) was reacted with the ferric chloride reagent of DE MOSS *et al.*[3,4] (2 ml) in water (7 ml) and the red coloration was immediately measured in a Hilger Uvispek at 540 mμ N-[2-Amino-3-(*p*-hydroxyphenyl)propionyl] hydroxylamine, prepared from tyrosine methyl ester and hydroxylamine[5], was employed as a reference substance (Table I).

A second sample of the peptidyl-nucleotidates was reacted with hydroxylamine in the same way and the hydroxamates then concentrated by chromatography on

TABLE II

HYDROXAMATES FORMED FROM YEAST EXTRACTS AT DIFFERENT STAGES OF GROWTH

a, alcoholic extract; b, trichloroacetic acid extract.

Growth period (h)	*RF value of hydroxamates with respect to the tyrosine derivative in butanol–acetic acid–water (4: 1: 1, v/v) on Whatman No. 3 paper*															
	0.64		*0.72*		*0.88*		*0.96*		*1.28*		*1.4*		*1.6*		*2.2*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.0	+						+		+	+			+			
1.0	+								+				+	+	+	+
2.0						+			+					+	+	+
3.0													+	+		+
4.0									+				+	+		+
5.0					+				+				+	+		+
5.6						+				+				+	+	
6.6					+										+	+
23.6					+								+	+		+
29.6			+								+		+	+	+	+
47.6						+					+		+	+	+	

TABLE III

DISTRIBUTION OF PEPTIDYL-NUCLEOTIDATES
FROM YEAST EXTRACTS AT DIFFERENT STAGES OF GROWTH

a, alcoholic extract; b, trichloroacetic acid extract.

Growth period (h)	*RF values in n-butanol–acetic acid–water (4: 1: 1, v/v) on Whatman No. 3 paper*																			
	0.022		*0.047*		*0.088*		*0.138*		*0.22*		*0.33*		*0.40*		*0.45*		*0.55*		*0.60*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.0		+	+					+	+									+		
1.0	+	+					+	+	+		+		+					+	+	
2.0	+		+			+	+	+	+	+	+	+	+		+			+	+	
3.0		+					+		+	+	+		+					+	+	
4.0	+						+	+	+	+	+		+					+	+	
5.0		+				+	+	+	+	+	+							+	+	
5.6	+	+				+	+	+	+		+		+						+	
6.6		+				+	+	+	+	+	+		+				+	+	+	
23.6	+	+				+	+	+	+		+	+	+		+		+		+	+
29.6	+						+	+	+		+	+	+		+	+			+	
47.6	+					+	+	+	+	+	+	+	+	+	+	+		+	+	

TABLE

COMPOSITION OF PEPTIDYL-NUCLEOTIDATE

a, alcoholic extract;

Amino acids	*Zones*													
	A		*B*		*C*		*D*		*E*		*F*		*G*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
α-alanine	+	+	+	+++	tr	+	+	+	+			+	+	+
Arginine	+	++	++	tr	+	++	+	tr	+				+	tr
Aspartic acid	+	+	+	tr			+		+			++		
Cystine	tr	++			tr			tr				tr		
Glutamic acid		++	+	+	+		+		+			++	+	
Leucine	+	tr	tr	+				++	tr			tr	+	+
Valine	+	tr	tr	+					tr				+	tr
Purines														
Adenine	+	+	+	+	+	+	+	+					+	
Guanine		+			+									
Hypoxanthine	+		+	+				+						
Xanthine		+												
Pyrimidines														
Cytosine		+			+									
Uracil	+	+	+	+	+	+	+	+	+			+	+	+

Whatman No. 3 paper in ethanol–2 N ammonia (9:1, v/v)[6] in which the hydroxamates moved as a broad band ($R_{\text{leucine}} = 1.27$) ahead of contaminating amino acids and peptides. The hydroxamates themselves were then resolved further by chromatography on Whatman No. 3 paper in butanol–acetic acid–water (4:1:1, v/v). Most of the hydroxamates had R_F values greater than those of the amino acid derivatives (α-alanine, 0.36; proline, 0.45; valine, 0.69; leucine, 0.88). Individual zones (Table II) were extracted with water, the solutions concentrated and the hydroxamates hydrolyzed by means of 6 N hydrochloric acid at 100° overnight. The resulting amino acids were examined by two-dimensional chromatography[6].

Examination of peptidyl-nucleotidate concentrates I

The peptidyl-nucleotidates were separated initially by chromatography in butanol–acetic acid–water (4:1:1, v/v) using Whatman No. 3 paper. The majority had R_F values ranging from 0.088–0.40 (*cf.* Table III) and although this permitted a moderate degree of separation, further resolution was required. Accordingly, they were separated by two-dimensional chromatography using first butanol–acetic acid–water (4:1:1, v/v), and then acetone–30% acetic acid (1:1 or 3:1, v/v). Their positions were revealed by means of the hydroxylamine-ferric chloride reagent[7] (Fig. 1).

In addition to peptidyl-nucleotidates the concentrate was examined for free amino acids[6] and purines and pyrimidines[8].

The peptidyl-nucleotidates, separated from amino acids, peptides, purines and pyrimidines on Whatman No. 3 papers by two-dimensional chromatography as above, were eluted individually with 0.05 N hydrochloric acid and the acid extracts hydrolyzed

IV

ZONES IN EXTRACTS OF EAST

, trichloroacetic acid extract.

(cf. Fig. 1)

H		*J*		*K*		*M*		*O*		*Q*		*S*		*T*	
a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
+	+	+	+	+	+	++	+	+		+		++	+		tr
+	+	+	++	+	tr	++	++	+		+		+	tr		+
++						++	tr								
tr						tr									
+++				+		++	+	+							
tr				+	tr	++									
tr				+	+	++									
+			+	+	+		+			+					
										+					
							+								
+	+	+	+	+	+	+	+	+		+		+	+		+

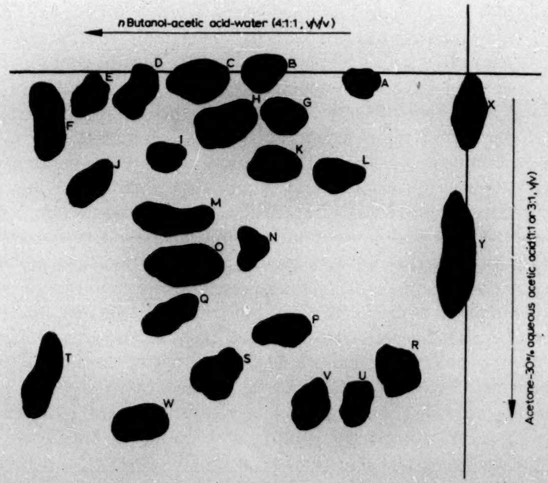
n Butanol-acetic acid-water (4:1:1, v/v/v)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Acetone-30% aqueous acetic acid (1:1 or 3:1, v/v)

Fig. 1. Diagrammatic representation of chromatogram of compounds reactive towards hydroxylamine. Reference zones; hypoxanthine (P); guanosine, xanthine, xanthosine (R); uracil (S); adenosine (U); adenine, cytosine, inosine (V); thymine (W); adenosine diphosphate, adenosine triphosphate, cytidylic acid (X); and adenylic acid, diphosphopyridine nucleotide, guanylic acid, thymidylic acid, uridine diphosphate glucose, uridylic acid (Y).

with 6 N hydrochloric acid as above. The hydrolyzates were freed of hydrochloric acid by distillation, finally repeatedly extracted with ethanol. The acid-free solutions were treated with Amberlite 1R120 (acid form, 5 g), the resin removed and amino acids and bases desorbed from the adsorbent by soaking it in 2 N ammonia solution for 48 h. The ammoniacal solutions were concentrated to dryness *in vacuo* and the residues individually chromatographed for purines and amino acids in solvent systems already described[6,8] (*cf.* Table IV).

The original effluents from the resin were concentrated *in vacuo* and the concentrates hydrolyzed by means of 72 % perchloric acid[9]. Excess perchloric acid was neutralized by means of potassium hydroxide, potassium perchlorate removed by filtration and the filtrate concentrated and chromatographed for purines and pyrimidines[8] (Table IV).

RESULTS AND DISCUSSION

The variation in total activated material is shown in Table I from which it may be seen that the pool of peptidyl-nucleotidates grows to a maximum 1 h after transfer of the yeast to fresh medium, thereafter declining during active growth of the yeast and subsequently increasing up to about 48 h. After this period autolysis apparently sets in rapidly and the amount of peptidyl-nucleotidate falls abruptly. Similar effects are observed in both the ethanolic and trichloroacetic acid extracts.

Although the rise in reactivity of the extracts towards hydroxylamine was striking, whether the results were calculated on the basis of unit dry weight or total weight of culture (Table I), the possibility existed that the bulk of the increase was due to only one or a few components of the mixture. However, chromatography of the extract (Tables II, III and V) revealed that this was not the case. The chromatographic pattern of materials reacting with hydroxylamine at all stages of growth of the yeast is shown in Fig. 1. It is noteworthy that the bulk of peptidyl-nucleotidates is well separated from most of the common purines and pyrimidines and derived nucleosides and nucleotides, which are located at the periphery of chromatograms prepared with the aid of the new solvent system. The variation in the chromatographic pattern is indicated in Tables II, III and V. From Table V particularly it may be seen that although the pattern varies from time to time, no one compound is predominant at any time. It was of particular interest that certain components or mixtures of components were present during both the early active growth phase and the later phases. It seems reasonable to assume, therefore, that these are intermediates in synthetic processes and that their synthesis continues even during the resting phase of the cell, presumably because they are not integrated further into protein. The accumulation of material reactive towards hydroxylamine after active growth of the yeast had ceased, would then be explicable as the result of the increase in amount of these particular peptidyl-nucleotidates among others.

In view of the above findings and of the isolation of individual peptidyl-nucleotidates of known composition from a 48 h culture of this yeast[1,8], the composition of the zones separated chromatographically (Fig. 1) at various stages of growth of the yeast was determined (Table V; *cf.* Table IV). As in the previously isolated compounds[1,8], the predominant amino acids were arginine and α-alanine and the only other amino acids detected were aspartic acid, cystine, glutamic acid, leucine and valine. The presence of only these amino acids in the active compounds was

TABLE V

VARIATION IN COMPOSITION OF MIXTURE OF PEPTIDYL-NUCLEOTIDATES IN EXTRACTS OF YEAST DURING GROWTH

* Zones were clearly u.v. light absorbing and gave strong reaction with hydroxylamine. + Zones absorbed u.v. light but reaction with hydroxylamine doubtful. a, alcoholic extract; b, trichloroacetic acid extract.

Growth period (h)	*Zones (cf. Fig. 1 and Table IV)*																																							
	A		*B*		*C*		*D*		*E*		*F*		*G*		*H*		*I*		*J*		*K*		*L*		*M*		*N*		*O*		*P*		*Q*		*S*		*T*		*W*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.0			*		*	*	*						*	*	*	+					*				+	*	+		+	+	+		+		+					
1.0					*	+	*	+		+				*	*	+				*	+	*			*	*							+					*		
2.0			*	*									*	*	*						*			*		*	+	*	+	+	+				+	+			+	
3.0		*	*	*	*	+	*	+	*	+		*		*	*	*			*	+	+	+				*			+									*	*	
4.0		*	*	*	*		+		+				*	*	+	*										*	+	*	+	+	+		+			*				
5.0		*		+		*	+						*	+	*	*					*				+	*			+	+										
5.6	+	+	+		+	+			+	+			*		*	+	*			*	*	*			+	*	+		+		+				+					
6.6					*	*	*	+					*			+				*	*				+	*	+		+											
23.6	+	*	+	*	*	+	*	+					*	*	*	+	*		+		+	*				+		*	+		+							*		
29.6			+		*	*	*		*				*	+	*	*			*	*	*	*			+	*					+							+	+	
47.6		+	*	*	*	+	*	+				+	*			+	*			*	*						+		+									*		
49.8							*					+			*					*	+					+				+								*		

confirmed by hydrolysis of the hydroxamates freed from contaminating amino acids and peptides by chromatography. Of the purines and pyrimidines the principal representatives were again uracil and adenine but cytosine and guanine were also detected as constituents of the zones A and C (Fig. 1). It is relevant to note that less well defined concentrates with acylating properties from guinea pig liver have recently been found to contain all the bases, including thymine[10], derived from the nucleic acids. This base has not been detected in the activated compounds from yeast, but small amounts of hypoxanthine and xanthine have been found. It is probable that these last compounds are degradation products formed by the action of perchloric acid on the bases originally present during hydrolysis.

The central position of uracil in the active compounds from yeast is quite clear. In view of the participation of this base uniquely in the structure of ribonucleic acid it appears probable that active compounds such as those described here form a link in the well-known relationship between ribonucleic acid or its precursors[11–14] and protein synthesis. This idea is supported by the predominance in the peptidyl-nucleotidates of the above-mentioned amino acids, which are common to nucleoproteins and indeed to ribonucleoprotein particles which participate in protein synthesis, *e.g.*, in rat liver[15]. The common occurrence with uracil of adenine in many of the yeast components suggests that di- or polynucleotides of the type found in the isolated compounds CE_1, TCA_1, TCA_2, D_1 and D_2 from a 48 h culture of the same yeast (*cf.*[1]) are present. Certainly, the first four of the above isolated compounds occupy the zones N, K, A, and B respectively in Fig. 1 of the present communication, these zones in each case being found to contain adenine, uracil and the amino acids expected. Incidentally, the compounds D_3 and D_4 previously described occupy zones P and M (Fig. 1) while arginylalanylarginylalanyl-5′-uridylate[8] is located in zone N. The presence in such peptidyl-nucleotidates of the dinucleotide having the 5′,3′-phosphodiester linkage[1] typical of ribonucleic acid lends weight to the suggestion that the activated compounds play a part in elaborating nucleoproteins. That other oligonucleotides may be present also is suggested by the presence together of further bases, *e.g.*, adenine, guanine, cytosine, and uracil in zones A and C.

The important role of uracil in peptidyl-nucleotidates perhaps provides a rationale for the earlier finding[16] that pyrimidine derivatives stimulate protein synthesis, *e.g.*, in particulate fractions from pea seedlings, and the more recent observation[17] that in a uracil-requiring mutant of *E. coli*, induction of β-galactosidase requires the pyrimidine. In connection with the present findings it is of especial interest that Okazaki and Okazaki[18] have shown that during unbalanced growth of *Lactobacillus acidophilus*, which is in some respects like that at the end of the logarithmic growth of yeast as above, large increases occur of the pool of uracil ribomononucleotide derivatives. When, however, the supply of uracil was depleted, the synthesis of both ribonucleic acid and protein was inhibited. Possibly, peptidyl-nucleotidates represent various stages in the synthesis not only of protein but of ribonucleic acid also (*cf.* Michelson[19]). Evidence that peptides and oligonucleotides, rather than ribonucleic acid itself, take part in protein synthesis at least, was adduced by Steinberg and Afinsen[20] who observed that, in the formation of ovalbumin, ribonucleic acid could be replaced by di- and trinucleotides. Moreover, Jordan and Innis[21] noted as a result of studying the action of the antibiotic vancomycin on the synthesis of protein in *S. aureus* that nucleic acid precursors can replace nucleic acid in effecting such synthesis. A similar conclusion is reached by Gorman and Halvorson[22] as a result of the finding that the rate of protein synthesis in *Pseudomonas azotogensis* does not necessarily parallel the rate of synthesis of nucleic acid, and by Reis, Coote and Work[23] from the failure of ribonuclease to inhibit incorporation of amino acids into the proteins of liver fractions. It is noteworthy that in some such systems, *e.g.*, the "lubrol-KCl" microsomes[24], incorporation of amino acid into protein occurs independently of the above-mentioned S-ribonucleic acid and in other systems, *e.g.*, extracts of bull spermatozoa[25], incorporation occurred in the absence of nucleic acid but in presence of oligonucleotides. It is perhaps significant in view of the amino acid composition of peptidyl-nucleotidates that the "lubrol-KCl" microsomes contain proteins particularly rich in basic amino acids[26].

Alternatively, the peptidyl-nucleotidates might be formed as a template composed of nucleic acid or oligonucleotide, presumably *via* activated amino acids. The peptidyl-uridylates would then represent the products formed by peeling of a peptide chain from the template bearing the uridylic acid residue at one end and scission of this residue from the nucleotide chain, perhaps by a ribonuclease. The presence of a latent ribonuclease, capable of activation by bases, in ribonucleoprotein particles from various organisms, including yeast[27], has been described by various workers (*cf.* Elson[28]; Zillig, Krone and Albers[29]). Similarly, the dinucleotide derivatives would be the products of splitting of the penultimate phosphodiester bond of a polynucleotide chain bearing the terminal dinucleotide. Whether or not the function of terminal uridylic acid in this respect is related to the rapid turnover of this terminal nucleotide in ribonucleic acid of thymocyte nuclei[30], in contrast to the stability of cytidylic acid, remains to be elucidated.

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THE ENZYMIC OXIDATION OF 2-CHLOROPURINE

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SUMMARY

2-Chloropurine is the first purine, for which simultaneous enzymic oxidation along two different pathways has been established. Attack at either carbon atom 6 or 8 proceeds at the same rate. This phenomenon is ascribed to the mesomeric (+M) effect of the halogen in position 2, which exhibits pronounced aromatic properties.

INTRODUCTION

Recently a hypothesis was advanced to explain the fact that the monohydroxy purines use only one of the two possible alternative pathways of enzymic oxidation leading to uric acid[1]. It was postulated that in the ES complex each derivative assumes a specific "activated" form in which a free NH-group at either position 3 or 9 participates in binding; the "active" form of the molecule then exposes one specific carbon atom to nucleophilic attack by a hydroxyl ion. However, application of this reaction scheme to purine itself met with difficulties because it was found that 7- or 9-methylpurine are also oxidized to the corresponding hypoxanthines. The presence of a free NH-group in the imidazol ring is therefore not essential for activation of carbon atom 6 in purine.

In contrast to purine, pteridine is attacked by mammalian xanthine oxidase (XO) simultaneously at carbon 2 and 4, the latter corresponding to position 6 in the purine ring[2]. The lack of enzymic attack at position 2 in purine may be ascribed to a more rigid localisation of the double bonds in the pyrimidine moiety, due to fixation of the C(4)–C(5) double bond, common to both heterocyclic rings. The requirement of 6 π-electrons for resonance stabilisation of the imidazol ring is being held responsible for this MILLS-NIXON-like effect[3]. In pteridine, fixation of the central double bond may be assumed to be less stringent, because of the mutually opposed polarity of the $\overset{6}{C}{=}\overset{5}{N}$ and $\overset{7}{C}{=}\overset{8}{N}$ groups, which detracts from the aromatic character of the pyrazine ring.

On this basis, it may be expected that introduction of polar substituents, which do not alter the basic unsaturated structure of purine, but induce a different distribution of the π-electrons, will make positions other than C-6 susceptible to enzymic attack. It has been observed previously that in the pyrimidine ring a strong electron-donating substituent, such as the 6-dimethylamino group, makes all available carbon

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Abbreviations: XO, xanthine oxidase; O.D., optical density.

[*] Part of a thesis, to be submitted to the Faculty of Science, The Hebrew University, Jerusalem (Israel).

atoms refractory[4,5]. On the other hand, 6-chloropurine is oxidised enzymically to 6-chloro-2,8-dihydroxypurine[6], although the pathway of this reaction has not been elucidated. As a suitable test case, we have selected 2-chloropurine (I) because the substituent in position 2 is relatively stable[7-9] and can be expected to survive during the enzymic oxidation. The results obtained in the reaction of I with XO may shed light on the mechanism of the oxidations, catalysed by this enzyme.

MATERIALS AND METHODS

Purine derivatives

2-Chloropurine[9] (I) and its 6-hydroxy derivative[10] (II) were a gift of Dr. J. A. MONTGOMERY, Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama, USA.

2-Chloro-8-hydroxypurine (III) was synthesized as follows: An intimate mixture of 2-chloro-4,5-diaminopyrimidine[11] (0.5 g) and urea (0.7 g) was heated for 25 min to 200–210°. The cake was extracted repeatedly with hot water. Upon cooling, clusters of colorless plates were deposited. Yield: 0.2 g (30 %); m.p. > 300°.

Anal. calcd. for $C_5H_3N_4OCl \cdot H_2O$: C, 31.9; H, 2.7 %. Found: C, 32.3; H, 2.5 %.

2-Chloro-6,8-dihydroxypurine (IV) is also a new compound. It was obtained as end-product of all enzymic reactions, studied in this paper. The u.v. spectrum of IV is very similar to that of 6,8-dihydroxypurine and is clearly distinguished from uric acid. IV and uric acid also show different R_F values (Table I). Therefore, there can be no doubt that the end-product of oxidation of I, II and III has retained the chlorine atom in position 2.

Enzymes

Highly purified XO from cow's milk was obtained through the courtesy of Professor F. BERGEL and Dr. R. C. BRAY of the Chester Beatty Institute of Cancer Research, London, England. This preparation, when diluted 1:4800, produced at 28° and pH 8.0 1 μg/ml/min of uric acid, while $6.5 \cdot 10^{-5}$ M xanthine served as substrate.

In all experiments, 10 units/ml of catalase (Worthington) was added. This enzyme concentration is capable of decomposing 10 mg/ml/min of H_2O_2, when the latter is approximately $1.5 \cdot 10^{-3}$ M.

Kinetic measurements

The progress of enzymic oxidation was followed at the appropriate wave lengths by the use of a Beckman u.v. spectrophotometer. The temperature was maintained at 28° by a dual thermospacer; 0.01 M phosphate of pH 8.0 served as buffer. Two types of controls were used simultaneously: One quartz cell, containing substrate and buffer only, and a second one, to which also catalase was added. Since none of the purines studied presently underwent any reaction in the absence of XO, the changes of O.D., taking place under the influence of this enzyme, were plotted directly as function of time and the initial rates calculated from the linear portions of the kinetic curves (see Figs. 2 and 4).

Chromatographic analysis

Sheets of Whatman paper No. 1 were first washed according to the procedure of

BERGMANN, DIKSTEIN AND CHAIMOVITZ[12] and then air-dried. The following solvents were used for development by the descending method:

Solvent A, 95 % ethanol–glacial acetic acid–water (85:5:10, v/v/v);

Solvent B, 95 % ethanol–pyridine–water (70:20:10, v/v/v);

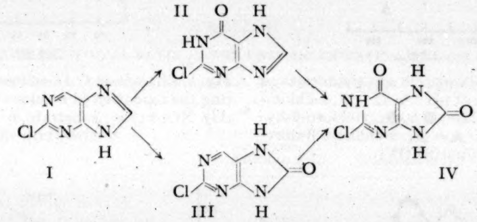
Solvent C, isopropanol–dimethylformamide–25 % ammonia (65:25:10, v/v/v). Spots were located by observing fluorescence under a Mineralight u.v. lamp, emitting light of about 255 mμ. They were then cut out and extracted with suitable buffers for determination of absorption spectra.

The pertinent data on the purines, used in this investigation, are collected in Table I. It is evident that replacement of the hydrogen in position 2 by chlorine has a bathochromic effect, which decreases with increasing number of hydroxyl groups in the molecule.

RESULTS

The enzymic oxidation of 6- and 8-hydroxy-2-chloropurine

The possible pathways of the oxidation of 2-chloropurine are indicated in the following scheme:



In order to evaluate the chances to isolate possible intermediates in the conversion of I to IV, it is necessary first to measure the susceptibility of the monohydroxy derivatives II and III to enzymic attack. The absorption spectra in Fig. 1 indicate that oxidation of 2-chloro-6-hydroxypurine to IV can be conveniently followed at 275 and 280 mμ. The changes of O.D., observed at these wave lengths, are plotted in Fig. 2 as function of time. The slope of the initial portion of each curve gave the same rate value. The oxidation of the 8-hydroxy derivative III, however, was too slow to be determined accurately. Therefore, only an approximate value, derived from measurements at 260 and 290 mμ, is given in Table I. The oxidation of the 6-hydroxy derivative is about 40 times more rapid than that of its 8-isomer. This result should be compared to the ratio of formation of uric acid from xanthine and 2,8-dihydroxypurine, respectively, which was determined previously as 500:1 (see ref. 13). The end-product of oxidation of II and III was found identical in every respect.

Oxidation of 2-chloropurine (I)

In Fig. 3, the absorption spectra of 2-chloropurine and its potential oxidation products are recorded. From these curves, 3 wave lengths were selected for rate determinations: (a) At 308 mμ, the isosbestic point of I and II, an initial increase in O.D. will measure the rate of formation of III; (b) at 255 mμ, the isosbestic point of

I and III, formation of II can be recognised by the initial rise in O.D.; (c) finally, at 235 mμ, the two potential intermediates in the oxidation of I possess an isosbestic point, and here any initial increase of O.D. is proportional to the total rate of disappearance of 2-chloropurine, regardless of the pathway used by XO. Fig. 4 represents the results of such measurements. The initial rate values, derived from Fig. 4 and shown in Table II, prove that both intermediates are formed at practically identical speed

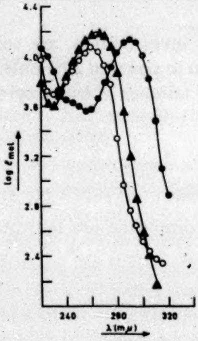


Fig. 1. Ultraviolet absorption spectra, measured in aqueous solution at pH 8.0. ○—○, 2-chloro-6-hydroxypurine (II); ●—●, 2-chloro-8-hydroxypurine (III); ▲—▲, 2-chloro-6,8-dihydroxypurine (IV).

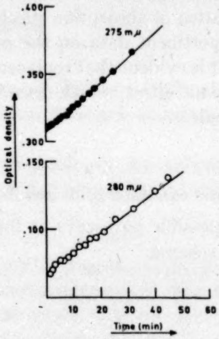


Fig. 2. Changes in O.D. as function of time during the oxidation of 2-chloro-6-hydroxypurine (II). XO, 1:800; substrate, $6 \cdot 10^{-5}$ *M*; pH 8.0; temperature 28°.

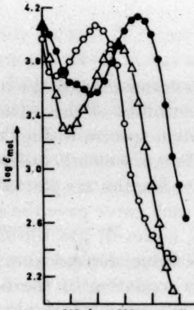


Fig. 3. U.V. absorption spectra of 2-chloropurine (I) and its monohydroxy derivatives at pH 8.0. △—△, 2-chloropurine (I); ○—○, 2-chloro-6-hydroxypurine (II); ●—●, 2-chloro-8-hydroxypurine (III).

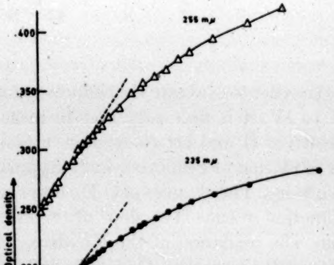


Fig. 4. Changes in O.D. during the enzymic oxidation of 2-chloropurine. XO, 1:1000; substrate, $6.5 \cdot 10^{-5}$ *M*; pH 8.0; temperature 28°. 235 mμ-isosbestic point of II and III (rate of consumption of 2-chloropurine). 255 mμ-isosbestic point of I and III (rate of formation of II). 308 mμ-isosbestic point of I and II (rate of formation of III).

TABLE I

PROPERTIES OF 2-CHLOROPURINE AND ITS HYDROXY DERIVATIVES

Values in parentheses refer to the non-chlorinated analogs and are recorded for comparison.

Compound	*λ_{max} (mμ) at pH 8.0*	*R_F values in solvent* A	B	C	*Fluorescence*	*Enzyme concentration used*	*Relative rate of oxidation* (xanthine = 100)*
2-Chloropurine (I)	275 (263)	0.83 (0.62)	0.78	0.73	Blue-violet	1:1000	3.1 (20)
2-Chloro-6-hydroxypurine (II)	259 (252)	0.63 (0.52)	0.37	0.44	Violet	1:800	0.8 (70)
2-Chloro-8-hydroxypurine (III)	290 (280)	0.78 (0.61)	0.80	0.66	Sky-blue	1:100	$<$ 0.02 (1.5)
2-Chloro-6,8-dihydroxypurine (IV)	265–266 (261)	0.37 (0.34)	—	0.42	Violet		— (100)
Uric acid	292	0.26	0.31	—	Dark-violet		—

* Calculated for an enzyme dilution of 1:4800.

TABLE II

EVALUATION OF INDIVIDUAL RATES IN THE ENZYMIC OXIDATION OF 2-CHLOROPURINE

Xanthine oxidase, 1:1000; 2-chloropurine, $6.5 \cdot 10^{-5}$ *M*; pH 8.0; temperature 28°.

	Wavelength observed 255 mμ (Formation of II)	308 mμ (Formation of III)	235 mμ (Disappearance of I)
(a) Observed initial change in O.D. (calculated per hour; from Fig. 4)	0.180	0.106	0.165
(b) Calculated change in O.D. per mole of substrate, $\times 10^{-4}$ (from Fig. 3)	0.70	0.40	0.315
(c) Initial rate = (a)/(b) (μmoles/h)	25.7	26.5	52.4
(d) Relative rate (%) (xanthine = 100)	1.49	1.53	3.08

and that indeed the rate of consumption of substrate I (column 4) is very nearly equal to the sum of the rates of formation of II (column 2) and III (column 3).

Chromatographic identification of the intermediates

The data in Table I demonstrate that both intermediates II and III are formed faster than they are consumed in the subsequent oxidation step. Therefore, they should accumulate in sufficient concentrations to be isolated and identified on a paper chromatogram. Unfortunately, no single solvent has been found that would separate all four components (I–IV) of the reaction mixture. Therefore, we had recourse to separation in two successive steps: First, by the use of solvent C, 2-chloropurine was separated from III and the latter from a mixture of II and IV (see Table I). This mixture alone was then rechromatographed in solvent A. The reaction with XO was interrupted in this case at an early stage (after 20 min) by immersion in boiling water for 10 min. The solution was then concentrated *in vacuo* and spotted on paper. The results shown in Fig. 5 and 6 and in Table II give final evidence that both II and III are present as intermediates. In view of the necessarily short duration of the enzymic reaction, the amount of IV present in this experiment was too small to be discovered by fluorescence in u.v. light. However, an extract of the appropriate zone on the chromatogram gave an absorption spectrum, identical with that of 2-chloro-6,8 dihydroxypurine (IV), isolated from enzymic reactions, which had proceeded to completion.

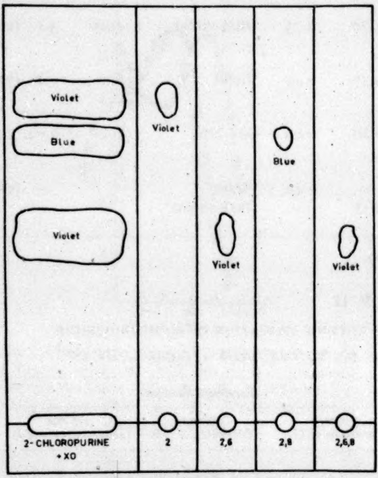


Fig. 5. Paper chromatogram of the reaction mixture, resulting from enzymic oxidation of 2-chloropurine. XO, 1:1000; substrate, $1.3 \cdot 10^{-4}$ *M*; pH 8.0; temperature, 28°. The reaction was stopped after 20 min. Development of the chromatogram with solvent C. Figures indicate position of substituents in the purine ring (2 always marks the chlorine atom). Note that the lowest spot on the left hand comprises a mixture of II and IV.

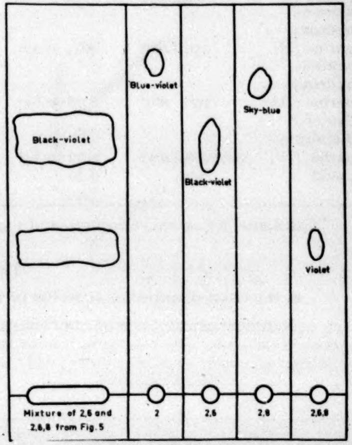
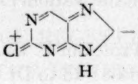


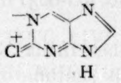
Fig. 6. Chromatographic separation in solvent A of a mixture of 2-chloro-6-hydroxypurine (II) and 2-chloro-6,8-dihydroxypurine (IV), resulting from the experiment in Fig. 5. Note that the lower spot on the left hand did not show u.v. fluorescence, but was identified by spectrophotometry of its aqueous extract.

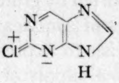
DISCUSSION

The fact that xanthine can convert anaerobically XO into its reduced form[14] shows that the oxygen atom, introduced into the purine nucleus by the enzymic process, originates from water. Therefore, we may assume that the carbon atom with the highest positive charge will combine with a hydroxyl ion and thereby decide upon the direction of the enzymic oxidation, the ability of a specific atom to serve as reaction center being determined by the polarity of the substrate in the enzyme substrate complex[1]. Previously, it was assumed that in the first step of the reaction addition of a water molecule to a C=N group does take place, followed by transfer of two hydrogen atoms to the prosthetic group of the enzyme. However, evidence has now been obtained to support the hypothesis that direct nucleophilic substitution by OH^- may lead to the enzymic oxidation product. For the following discussion, however, this point is irrelevant.

The low reactivity of chlorine in position 2 of various purines[7–9] can be explained on the same basis as the stability of the halogen in chlorobenzene: The C–Cl bond possesses partial double bond character, due to contributions of structures such as Ia, Ib and Ic, formulated on the assumption that the tautomeric hydrogen in the imidazol ring is located mainly at the 9-position, like in purine itself[15]. Among these three resonance forms, only Ia and Ib contain the triad system $\overset{3}{N} = \overset{4}{C} - \overset{9}{N}H$, which presumably attaches itself to an appropriate receptor group in the active center of XO[1]. Now it is evident that in Ia only C-6 may be attacked by a hydroxyl ion, C-8 being incapacitated by its formal negative charge. Similarly, in Ib only C-8 may serve as center for nucleophilic attack by OH^-, C-6 no longer participating in a polarisable C=N group, but being close to the negatively charged N-1 atom.

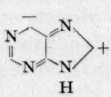

Ia

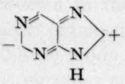

Ib

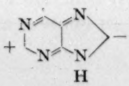

Ic

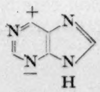
The fact that 2-chloropurine is attacked by XO at C-6 and C-8 with practically identical rates, may now be interpreted in the following way: In the ES complex, the polarities contributed by Ia and Ib are of approximately equal importance. A hydroxyl ion, approaching the purine nucleus, does therefore have equal chances to become attached to either position 6 or 8.

The above considerations may also shed light on the behavior of purine itself towards XO. In purine, no polar substituent is present to induce special distributions of the π-electrons. *E.g.*, on account of C-8 becoming a positive center, either C-6 or C-2 must assume a negative charge (see Va and Vb). Similarly, a positive charge at C-2 will place a negative pole on C-8 (Vc). Only carbon atom 6 is able to use the intrinsic polarity of the two C=N groups in the pyrimidine ring for the electron distribution, indicated in Vd, where the opposite charges are sufficiently far apart to permit the approach of OH^- to position 6. In addition, Vd is the only resonance hybrid, in which the central double bond is undisturbed and the aromatic character of the imidazol ring preserved. This resonance form, therefore, makes the greatest contribution to the structure of purine in the activated enzyme substrate complex.


Va


Vb


Vc


Vd

It is evident that the "active" form Vd does not possess the characteristic triad group $\overset{3}{N} = \overset{4}{C} - \overset{9}{N}H$. This is in accord with our previous observation that methylation of either N-7 or N-9 in purine does not prevent enzymic oxidation[1].

The observations presented here invite a study of other purines, bearing a single polar substituent in various positions. These experiments will be reported in due course.

ACKNOWLEDGEMENTS

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METABOLIC ALTERATIONS AFTER TOTAL BODY DOSES OF X-RADIATION

I. THE ROLE OF REGENERATING LIVER NUCLEI AND CYTOPLASM IN THE INHIBITION DUE TO X-RADIATION OF INCORPORATION OF TRITIUM-LABELED THYMIDINE INTO DNA

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SUMMARY

The effect of X-radiation on the incorporation of [^{3}H]TDR into the DNA of nuclei of reconstituted regenerating liver homogenates and into purified DNA incubated with irradiated and non-irradiated regenerating liver cytoplasm was studied. The results indicate that both the nuclei and the cytoplasm are affected by X-radiation but the effect is more marked on the cytoplasm than on the nucleus. These findings suggest that X-radiation interferes with the activity of the enzymes involved in the incorporation of nucleosides or nucleotides into the DNA.

INTRODUCTION

Inhibition of the incorporation of [^{3}H]TDR into the DNA of 30-h regenerating liver homogenates[1] obtained from rats irradiated with X-ray 6 h before sacrifice, suggests that the inhibitory effect occurs in the final steps of DNA biosynthesis, either through alteration of the primer or by interfering with the activity of the cytoplasmic enzymes involved in the synthesis of DNA. For all practical purposes, all the phosphorylating enzymes and polymerase activity can be recovered in the cytoplasmic fraction[2], and all the DNA can be found in the nuclear pellet[3] obtained after differential centrifugation of liver homogenate. Thus it is possible to study separately the effect of X-radiation on the activity of the cytoplasmic enzymes and on the nuclei, simply by recombining regenerating liver nuclei obtained from irradiated rats with regenerating liver cytoplasm obtained from non-irradiated animals, and *vice versa*. The purpose of this paper is to study the incorporation of [^{3}H]TDR in such reconstituted homogenates.

METHODS

Sprague-Dawley rats, weighing 180–200 g, were used in these experiments. The animals were hepatectomized according to the procedure of HIGGINS AND ANDERSON[4].

Abbreviations: [^{3}H]TDR, tritium labeled thymidine; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

The method of irradiation has already been described[1]. Tissue preparations were incubated according to the method of BOLLUM AND POTTER[3], slightly modified as described[1]. DNA was extracted, separated from RNA, and prepared for determination of specific activity[6]. Despite the fact that a self-absorption curve was determined for extracted [^{3}H]DNA, great care was taken to plate identical amounts of DNA in all experiments, and thus only minor corrections had to be applied. All incubations as well as all colorimetric and specific activity determinations were done in duplicate. In those experiments where nuclei and cytoplasm were recombined, 6 animals were hepatectomized for each experiment. 24 h later half the rats were irradiated with 1500 R and at 30 h or 48 h after hepatectomy all were sacrificed by decapitation. Livers from non-irradiated and from irradiated animals were pooled separately and prepared as 20 % homogenates in 0.25 *M* sucrose; the homogenates were then centrifuged at 600 × *g* for 10 min (4°). The nuclear pellet was washed 4 times with cold sucrose, but only the original cytoplasmic extract and the first washing were used to prepare the recombined homogenates or for incubation with purified DNA. Cytochrome oxidase, acid phosphatase and glucose-6-phosphatase activities and nitrogen were determined on the nuclear pellet by methods described previously[7] for the purpose of evaluating cytoplasmic contamination. The enzyme activities in the nuclear pellet never exceeded 5 % of the total activity in the whole homogenate. Conversely the DNA content of the cytoplasmic fraction[7] did not exceed 1 % of the total DNA. Purified DNA, to be used for comparison with whole nuclei, was prepared by the method for small scale preparation described by CHARGAFF[8] from the nuclear pellet (10 livers) washed 3 times with 0.25 *M* sucrose and twice with the following solution: Triton × 100, 0.001 %; sodium chloride 0.09 %; and trishexamethylene tetramine 0.05 *M*, pH 7.4.

REPORTS

The inhibitory effect of whole body irradiation on *in vitro* incorporation of [^{3}H]TDR into the DNA of 30 h regenerating livers is shown in Fig. 1. Inhibition was observed at all doses of X-radiation and the inhibition increased with the dose. While the individual variations in response to the administration of 350 R are considerable, variability is reduced at higher dose levels. It was therefore decided to employ higher dosages in the experiments with reconstituted homogenates. The data given in Fig. 1 are in good agreement with previous studies on incorporation of [^{14}C]orotic acid[6] and [^{3}H]TDR after X-radiation[1]. The incorporation of [^{3}H]TDR into nuclear DNA in the three types of reconstituted homogenates is presented in Table I for 30-h and 48-h regenerating livers respectively, after three different radiation doses. In Fig. 2 the same data are expressed as percentages of [^{3}H]TDR incorporation into DNA in homogenates reconstituted with non-irradiated regenerating liver nuclei and cytoplasm. It is clear that the incorporation of [^{3}H]TDR is inhibited whenever the reconstituted homogenate is made partly or entirely with constituents obtained from irradiated animals. Inhibition increases with the dose administered and it tends to be proportional to the logarithm of the dose. Inhibition is greater in those homogenates obtained from animals sacrificed 24 h after irradiation (48-h regenerating livers) than in those obtained from animals sacrificed 6 h after irradiation (30-h regenerating livers) when homogenates reconstituted under similar conditions are compared (Table I and Fig. 2). In both groups the incorporation of [^{3}H]TDR is inhibited to a greater extent when the reconstituted homogenate is prepared with regenerating liver nuclei from non-irradiated and regenerating liver cytoplasm from irradiated animals than in homogenates reconstituted with nuclei obtained from irradiated animals and cytoplasm obtained from non-irradiated animals. The inhibitory effect of X-radiation on the activity of cytoplasmic enzymes concerned in DNA synthesis is confirmed by the results presented in Figs. 3 and 4. In these experiments the incorporation of [^{3}H]TDR into purified DNA was measured in presence of increasing amounts of regenerating liver cytoplasmic extract obtained from animals irradiated with 1500 R, 24 h after partial hepatectomy and sacrificed 6 or 24 h later.

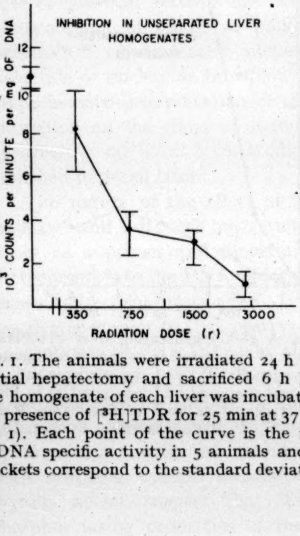

Fig. 1. The animals were irradiated 24 h after partial hepatectomy and sacrificed 6 h later. The homogenate of each liver was incubated in the presence of [^{3}H]TDR for 25 min at 37° (see ref. 1). Each point of the curve is the mean of DNA specific activity in 5 animals and the brackets correspond to the standard deviations.

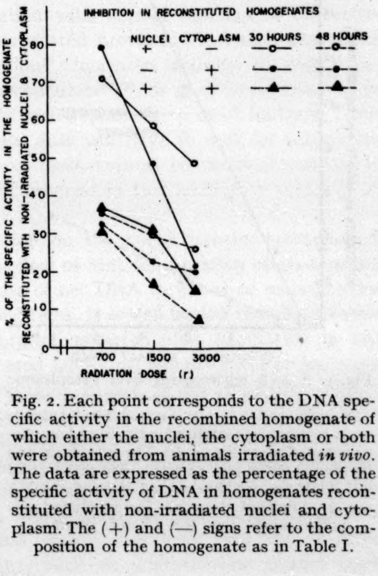

Fig. 2. Each point corresponds to the DNA specific activity in the recombined homogenate of which either the nuclei, the cytoplasm or both were obtained from animals irradiated *in vivo*. The data are expressed as the percentage of the specific activity of DNA in homogenates reconstituted with non-irradiated nuclei and cytoplasm. The (+) and (—) signs refer to the composition of the homogenate as in Table I.

TABLE I

INCORPORATION OF [^{3}H]TDR INTO DNA OF RECONSTITUTED HOMOGENATES

All animals were irradiated 24 h after partial hepatectomy and sacrificed either 30 or 48 h after the operation. The recombined homogenates were incubated for 24 min at 37°. The (+) sign, indicates that the cell fraction used in the reconstituted homogenates was prepared from livers of irradiated rats, the (—) sign indicates that it was prepared from livers of non-irradiated rats. Counts × 10^3/min/mg DNA.

Composition of the homogenate		*700 R*		*1500 R*		*3000 R*	
Nuclei	*Cytoplasm*	*30 h*	*48 h*	*30 h*	*48 h*	*30 h*	*48 h*
—	—	7,000	9,700	7,100	11,180	7,600	9,400
—	+	4,950	3,480	4,100	3,500	3,700	2,560
+	—	5,500	3,240	3,400	2,640	1,560	2,060
+	+	2,720	2,800	2,200	1,680	1,190	700

The incorporation of [^{3}H]TDR into purified DNA in presence of cytoplasm from livers of irradiated rats was compared with the incorporation of [^{3}H]TDR into purified DNA in presence of non-irradiated regenerating liver cytoplasm. The incorporation of [^{3}H]TDR was found to be markedly inhibited by X-radiation, both when the animals were sacrificed 6 h (30-h regenerating livers) after X-radiation and 24 h (48-h regenerating livers) after X-radiation.

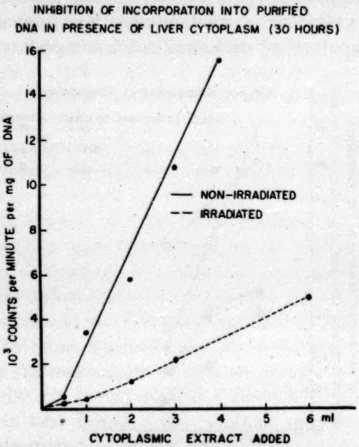

Fig. 3. A 30-h regenerating liver cytoplasmic extract of irradiated (5.6 mg of nitrogen/ml) or non-irradiated (5.65 mg of nitrogen/ml) animals was prepared by centrifuging the liver homogenate to sediment the nuclei. The cytoplasm was incubated for 25 min at 37° in the presence of [^{3}H]TDR (except that 0.5 mg of purified DNA was used instead of nuclei). The irradiated animals received 1500 R 6 h before sacrifice.

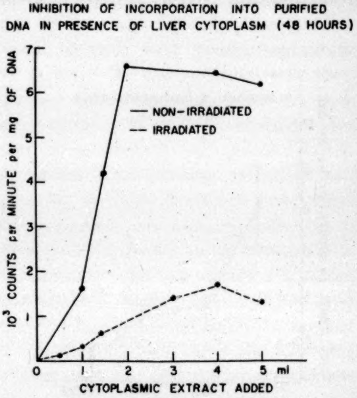

Fig. 4. A 48-h regenerating liver cytoplasmic extract of irradiated (4.72 mg of nitrogen/ml) or non-irradiated (4.65 mg nitrogen/ml) animals was prepared and incubated as in Fig. 3. The irradiated animals received 1500 R 24 h before sacrifice.

DISCUSSION

The inhibition of DNA synthesis in regenerating liver[9–11] or other actively growing tissues[12–21] by X-radiation has been demonstrated in numerous laboratories with a variety of precursors. It appears that X-radiation affects the incorporation of precursors into DNA more than it affects the incorporation of precursors in other major cellular constituents, such as, RNA[6,22] or protein[23]. Our studies were, therefore, mainly concerned with the elucidation of the mechanism of interference of X-radiation with DNA biosynthesis. This effect on both cellular constituents is not an isolated observation. Indeed, various investigators have made similar observations by studying the effect of u.v. or X-radiation on preparations ranging from amebae[24] to isolated mammalian[25] nuclei. Incorporation into DNA is more inhibited into reconstituted homogenates in which the cytoplasmic fraction was prepared from irradiated animals, than in those in which the nuclear fraction came from irradiated animals. The effect on the cytoplasm suggests that X-radiation markedly reduces the activity of one or more enzymes involved in the utilization of nucleotides or nucleosides for DNA biosynthesis. It is however not known whether X-radiation directly affects phosphorylating enzymes or polymerase, either by interfering with their biosynthesis, or by inactivating the enzyme. Indeed POLARD AND WITHMORE[26] explain the inhibition of antigen formation after irradiation of *E. coli*, by interference with their biosynthesis. The inhibition of carboxypeptidase[27] activity is ascribed to the denaturation of the protein by X-radiation. The oxidation of the SH group has been suggested to explain the inhibition of numerous SH enzymes by X-radiation[28]. It is thus possible that in our experiments X-radiation interferes with the active centers of the cytoplasmic enzymes involved in DNA synthesis.

Furthermore, interference of X-radiation with normal function of numerous other biochemical systems has been demonstrated previously and such alterations as uncoupling of oxidative phosphorylation[29], increased activity of deoxyribonuclease[30–32], ribonuclease[33], adenosinetriphosphatase[34, 35], or of enzymes involved in nucleotide or nucleoside breakdown[36] might well explain the present findings. Thus, comprehensive interpretation of the present data will have to wait for further investigation on the effect of X-radiation on these various biochemical systems in regenerating rat liver. Such studies are in progress in this laboratory and will be published in detail later.

The nature of the effect of X-radiation on the nuclei remains unexplained. LEHMAN[37] and BOLLUM[38] have studied the effect of heat denaturation on its capacity to act as a primer and report an increase of net DNA synthesis or incorporation of precursors into the DNA under such conditions. It is well known that X-radiation causes DNA denaturation[39, 40]. If denaturation occurred after irradiation *in vivo* to a degree similar to that observed in heated DNA, one would expect an increased incorporation of [^{3}H]TDR under these conditions. In fact, *in vivo* irradiation inhibits the incorporation of [^{3}H]TDR into irradiated nuclei incubated with normal cytoplasm. If, as suggested by LEHMAN[37], macromolecular alteration of DNA is needed for it to function as primer, the decreased incorporation after irradiation may be caused by interference with the modification of the macromolecule which accompanies DNA synthesis. CREASY AND STOCKEN[41], who studied the effect of X-radiation on thymus nuclei, suggest that X-radiation mainly interferes with the oxidative phosphorylating properties of the nucleus. Such an interpretation cannot apply to our findings; indeed, according to the same authors liver nuclei are not capable of oxidative phosphorylation.

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METABOLIC ALTERATIONS AFTER TOTAL BODY DOSES OF X-RADIATION

II. INCORPORATION OF DEOXYCYTIDYLIC AND THYMIDYLIC ACID INTO PURIFIED DNA AND NUCLEI IN PRESENCE OF REGENERATING-LIVER SUPERNATANT

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SUMMARY

The incorporation of [^{3}H]deoxycytidylic acid and [^{3}H]thymidylic acid into purified deoxyribonucleic acid was measured in the presence of regenerating liver supernatant obtained at various time intervals after partial hepatectomy. The animals were sacrificed either 6 h or 24 h after X-radiation. The results suggest that X-radiation interferes with the biosynthesis of the enzymes involved in the last steps of deoxyribonucleic acid synthesis. The effect of X-radiation on the nucleus was also investigated by studying the incorporation of [^{3}H]deoxycytidylic acid into the deoxyribonucleic acid of regenerating liver nuclei (48 h after partial hepatectomy) obtained from animals sacrificed 24 h after the administration of 1500 R *in vivo*. The incorporation of the precursor was found to be considerably reduced by X-radiation.

INTRODUCTION

In vivo X-radiation of partially hepatectomized rats inhibits incorporation of tritium labeled thymidine into DNA of homogenates[1]. X-radiation affects both the nucleus and the cytoplasm, but the nature of the alteration in each case remains unknown[2]. The aim of the present study was to investigate the effect of X-radiation on both the priming capacity of the nucleus and the activity of the cytoplasmic enzymes involved in the last steps of DNA synthesis in a system easier to control than homogenates. Therefore, the incorporation of [^{3}H] DCMP or of [^{3}H]TMP into purified DNA and into regenerating liver nuclei in presence of regenerating rat liver supernatant was measured.

Abbreviations: DNA, deoxyribonucleic acid; DCMP, deoxycytidylic acid; TMP, thymidylic acid; RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

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METHODS

Sprague-Dawley rats weighing 180 g were used. The methods of irradiation and partial hepatectomy have been described[1]. The nuclei used for incubations were prepared by differential centrifugation of a 5 % homogenate at 600 × *g* for 10 min, the nuclear pellet was washed twice in sucrose and checked for mitochondrial or microsomal contaminants[2]. Regenerating liver supernatant was prepared by centrifuging a 5 % homogenate (in 0.25 *M* sucrose) at 105, 400 × *g* for 60 min in the model L Spinco centrifuge equipped with rotor No. 40. The supernatant was checked for mitochondrial and microsomal contaminants by the determination of cytochrome oxidase and glucose-6-phosphatase activity[3]. 2 % cytochrome oxidase and 12 % glucose-6-phosphatase activity remained associated with the latter fraction. Regenerating liver cell fractions, were prepared by differential centrifugation of the homogenate. The nuclei were separated at 600 × *g* for 10 min, the small and large mitochondria were sedimented in a single pellet by centrifuging at 25,000 × *g* for 10 min and the microsomal pellet was sedimented at 105,400 × *g* for 60 min. Each fraction and a sample of the total homogenate were analyzed for nitrogen, DNA, RNA and for cytochrome oxidase, glucose-6-phosphatase, acid phosphatase, deoxyribonuclease, β-glucuronidase activities. The methods of analysis were the same as those used in previous studies[3] except for deoxyribonuclease determinations which were done at pH 5 in presence of 0.05 *M* acetate buffer. The creatine kinase used for the incubations was prepared according to the method of NODA *et al.*[4]. The results of the incorporation studies were, however, similar when pyruvic kinase and pyruvic phosphate (purchased from Sigma) were used as high energy phosphate generators. [^{3}H]DCMP was obtained from Schwartz Laboratories (0.93 C/m mole).

The labeled thymidylic acid used in these experiments was prepared from tritium labeled regenerating rat liver DNA. Partially hepatectomized animals were injected intraperitoneally with [^{3}H]thymidine 18, 23 and 26 h after operation and were sacrificed 6 h later. The nuclear pellet was prepared from liver homogenates[2]. After purification the extracted DNA[5] was treated with crystalline deoxyribonuclease and snake venom phosphodiesterase free of 5′ nucleotidase[6]. The nucleotides so obtained were then separated by the method of CANELLAKIS *et al.*[7]. Except when stated otherwise, the incubation methods were those described by MATSAVINOS AND CANELLAKIS[8]. The incubation was stopped by addition of perchloric acid to the mixture to reach a final concentration of 0.06 *M*. After repeated washings with cold 5 % perchloric acid, alcohol and Bloor's solution, the nucleic acids were extracted at 95° with a solution containing 10 % sodium chloride and 0.5 *M* Tris buffer. The DNA was separated from RNA by the method of SCHMIDT AND THANNHAUSER[9]. After precipitation of the nucleic acids with alcohol the contaminating RNA was hydrolysed by heating the nucleic acid preparation at 80° in 0.1 *N* sodium hydroxide. The DNA was then precipitated with 2 *N* hydrochloric acid and washed with alcohol. These procedures were repeated until the products of the sodium hydroxide hydrolysis were free of orcinol reacting substances. After several washings with cold alcohol, the purified DNA pellet was dissolved in 0.1 *M* ammonium hydroxyde and the concentration of deoxyribose was determined by the diphenylamine method[10]. Great care was taken to plate[1] identical amounts of DNA (0.08 to 0.1 mg when nuclear DNA and 0.06 to 0.07 mg when purified DNA was used). Therefore, only minor corrections for self-absorption had to be introduced. Self-absorption curves for increasing concentration of $[^3H]$ DCMP and DNA labelled with deoxycytidylic acid and thymidylic acid were prepared.

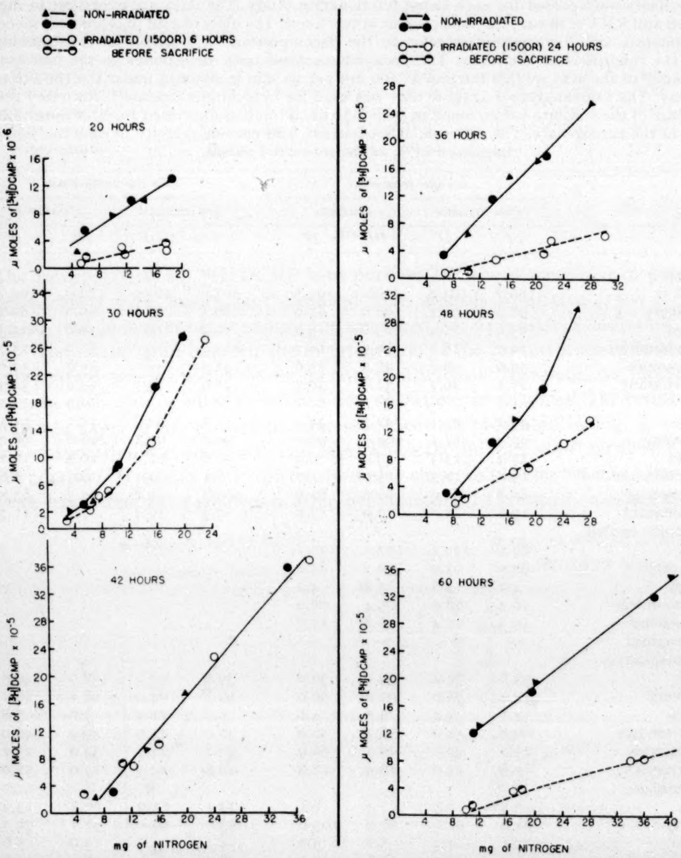

Fig. 1. The animals received 1500 R, 12, 14, 24, and 36 h after hepatectomy and were sacrificed 6 or 24 h later as indicated on the graph. Two separate experiments were done at each time sequence. The results of each experiment are plotted with different symbols. The time in hours refers, in each case, to the time interval between hepatectomy and sacrifice. For each experiment 3 normal and 3 irradiated livers were pooled separately. 10 ml of the incubation mixture B described by Canellakis and Matsavinos[8] was used for each test. The primer was Worthington DNA boiled for 10 min[17,18]. The preparation was incubated for 60 min at 37°. The data are expressed in μmoles of $[^3H]$DCMP incorporated /mg of nitrogen present in the regenerating liver supernatant added.

TABLE I

Two tissue fractionation studies were done at 20 and 36 h after partial hepatectomy. In each experiment tissue fractions of non irradiated animals (I, II) were compared with tissue fractions of irradiated animals (III, IV). The latter received 1500 R, 6 h before death when sacrificed 20 h after hepatectomy, or 24 h before death when sacrificed 36 h after hepatectomy. Two regenerating livers were pooled for each tissue fractionation study. The data are expressed in mg of nitrogen and RNA or in enzyme units present in two livers. The units of acid phosphatase, glucose-6-phosphatase and β-glucuronidase refer to the decomposition of 1 μmole of substrate /min under the conditions of the assay. The deoxyribonuclease unit corresponds to the increase in absorbancy of the acid soluble fraction at 260 mμ per 15 min incubation under the condition of the assay. The COOPERSTEIN-LAZAROW unit was used for cytochrome oxidase[19]. Recovery refers to the sum of the absolute values found in each individual fraction expressed in % of the absolute values in the homogenate. The nitrogen, RNA content, and enzyme activity in each fraction are expressed in % of the recovered values.

	20 h after hepatectomy				*36 h after hepatectomy*			
	Non irradiated		*Irradiated*		*Non irradiated*		*Irradiated*	
	I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
Nitrogen								
Total	189.2	193.4	190.2	194.5	205.0	203.0	187.0	194.2
Recovery	98.0	94.5	96.0	97.0	89.5	97.0	96.0	89.1
Nuclei	15.6	16.4	13.6	15.0	16.4	18.2	18.8	31.5
Mitochondria	18.7	18.0	19.0	18.7	13.2	19.7	11.0	15.7
Microsomes	38.6	36.4	36.0	35.6	41.5	39.0	35.2	37.0
Supernatant	27.1	29.2	31.0	30.7	28.9	23.1	35.0	32.3
RNA								
Total	45.2	43.4	42.3	44.2	71.2	62.7	71.2	61.7
Recovery	97.5	91.5	102.0	99.0	99.0	97.1	98.0	96.2
Nuclei	12.4	13.5	13.0	9.0	16.8	21.0	16.8	16.2
Mitochondria	13.0	12.5	13.0	7.0	8.4	9.2	8.0	8.6
Microsomes	60.8	62.0	62.0	62.0	67.5	63.6	67.5	63.5
Supernatant	13.8	12.0	12.0	12.0	7.3	6.2	7.7	11.7
Cytochrome oxidase								
Total	180.0	170.2	167.2	172.1				
Recovery	95.0	93.0	90.5	84.0				
Nuclei	4.2	4.6	3.8	4.2				
Mitochondria	76.3	78.0	69.4	78.0				
Microsomes	19.5	17.4	26.8	17.8				
Supernatant	—	—	—	—				
Acid phosphatase								
Total	33.6	32.2	33.0	30.0	37.9	45.2	38.0	40.5
Recovery	94.0	95.0	92.0	96.0	92.1	94.0	96.1	89.2
Nuclei	4.1	4.2	4.5	4.1	6.7	11.4	7.6	7.8
Mitochondria	60.0	59.0	58.7	59.0	47.0	44.0	40.4	46.0
Microsomes	23.0	22.8	20.3	18.9	25.7	24.7	34.0	25.2
Supernatant	12.9	14.0	16.5	18.0	20.6	19.9	17.9	21.0
Glucuronidase								
Total	8.5	8.3	7.4	8.1	12.1	14.0	14.2	13.3
Recovery	94.0	91.0	92.0	105.0	93.0	94.0	97.1	96.3
Nuclei	3.1	4.2	5.6	5.2	4.2	4.5	3.9	5.6
Mitochondria	51.1	48.0	52.2	47.1	48.0	51.2	58.0	43.6
Microsomes	21.2	23.1	26.0	19.3	20.1	23.9	19.6	24.5
Supernatant	24.6	26.5	16.2	28.4	27.7	20.4	18.5	26.3
Deoxyribonuclease								
Total	35.4	37.6	36.1	37.0	56.0	58.2	49.4	57.0
Recovery*	120.0	110.0	115.0	125.0	124.0	125.1	120.	110.
Nuclei	3.3	4.3	2.6	6.5	4.1	2.4	5.2	3.4
Mitochondria	43.5	42.5	42.5	46.5	39.0	41.2	48.6	44.5
Microsomes	20.0	19.4	20.6	16.0	27.2	24.3	19.1	18.1
Supernatant	33.2	33.8	34.5	31.0	29.7	32.1	27.1	34.

* Those recoveries greater than 100 % have been discussed previously[3].

Continued TABLE I

	20 h after hepatectomy				*36 h after hepatectomy*			
	Non irradiated		*Irradiated*		*Non irradiated*		*Irradiated*	
	I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
Glucose-6-phosphatase								
Total	92.0	88.2	94.0	96.4	117.0	142.0	126.3	124.3
Recovery	96.1	91.5	94.5	99.0	96.0	94.0	93.0	98.0
Nuclei	5.1	3.3	3.7	3.4	2.6	2.0	2.1	4.5
Mitochondria	2.9	2.7	2.7	3.1	10.8	10.0	8.7	11.2
Microsomes	86.0	86.5	88.0	81.5	82.0	86.8	86.0	80.2
Supernatant	6.0	7.5	5.6	6.0	4.6	1.2	3.2	4.1

RESULTS

The incorporation of [^{3}H] DCMP into purified DNA was measured in presence of regenerating liver supernatant obtained from animals irradiated (1500 R *in vivo*) 14, 24, and 36 h after hepatectomy and sacrificed 6 or 24 h after X-radiation.

The relationship between the incorporation of the precursor and the amount of supernatant present in the system is not strictly linear; therefore, the incorporation was, in each case, studied at various concentrations of enzymes. The results of two separate experiments at various time sequences are presented in Fig. 1. Inhibition of incorporation was observed in all cases where the animals were sacrificed 24 h after X-radiation. It makes little difference under those conditions whether the animals were irradiated 14, 24, or 36 h after hepatectomy. In contrast, marked inhibition

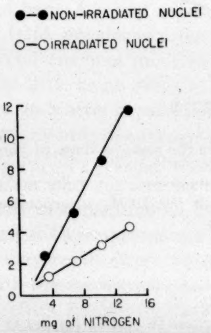

Fig. 2. The animals received 1500 R. 24 h after partial hepatectomy and were sacrificed 24 h later. The incubation mixture was the same as for Fig. 1. The nuclei of 3 non-irradiated and 3 irradiated regenerating livers were pooled separately. The DNA content of the nuclear preparation used for each test was 0.6 mg when nonirradiated nuclei and 0.58 mg when irradiated nuclei were used. The preparation was incubated at 34° for 25 min. The data is expressed as in Fig. 1.

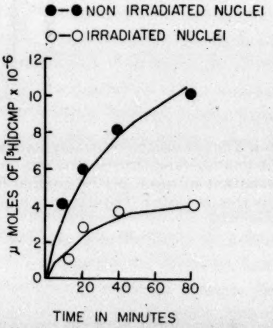

Fig. 3. The conditions of irradiation and incubation were the same as in Fig. 2 except that the enzyme concentrations were maintained constant (7.8 mg of regenerating liver supernatant nitrogen per incubation flask). The amount of the nuclear preparation was increased and the data are expressed in μmoles of [^{3}H]DCMP incorporated /mg of DNA added to the incubation flask.

was only observed in those animals irradiated 14 h after hepatectomy when the animals were sacrificed 6 h after irradiation.

The incorporation of [^{3}H]TMP into purified DNA in presence of regenerating liver supernatant obtained from animals irradiated 14 and 24 h after hepatectomy and sacrificed 6 h after X-radiation was also investigated. The results were identical to those obtained with [^{3}H]DCMP and therefore they are not reported in detail here.

The incorporation of [^{3}H]DCMP into non-irradiated and irradiated (1500 R *in vivo* 24 h after hepatectomy) regenerating liver nuclei was determined in presence of regenerating liver supernatant under conditions where the concentration of enzyme, the concentration of DNA or the duration of incubation were altered. In these experiments the animals were sacrificed 24 h after X-radiation. The results are plotted in Figs. 2, 3 and 4, and they clearly demonstrate that irradiated nuclei incorporate less of the precursor than non-irradiated.

The results of two tissue fractionation studies of regenerating liver homogenates obtained from animals irradiated 12 h after hepatectomy and sacrificed 6 or 24 h after X-radiation are presented in Table I. They show that X-radiation has no effect on total quantity and intracellular distribution of the various components investigated.

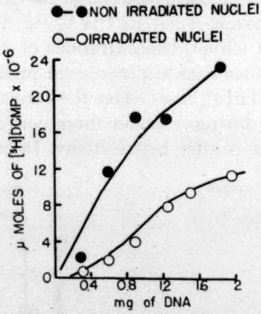

Fig. 4. The conditions of irradiation and incubation were the same as those of Figs. 2 and 3. The DNA (0.6 mg) and the regenerating liver supernatant concentration (7.8 mg of regenerating liver supernatant nitrogen per incubation flask) were maintained constant. Only the duration of the incubation is varied. The data are expressed in μmoles of [^{3}H]DCMP incorporated after various incubation times.

DISCUSSION

These experiments confirm previous findings *in vivo*[11, 12] and *in vitro*[12, 13] where it was demonstrated that X-radiation inhibits DNA synthesis in regenerating rat liver. Thus, the present work establishes a continuous base line of information between the *in vivo* situation and a simple *in vitro* system. It was also established that inhibition of DNA synthesis is due, altogether, to a decrease of the activity of the cytoplasmic enzymes involved in the last steps of DNA biosynthesis and to a decrease of the priming capacity of irradiated nuclei[2]. The reduced incorporation of [^{3}H]DCMP in presence of purified DNA and regenerating liver supernatant obtained from irradiated animals sacrificed 24 h after irradiation (Fig. 2) reflects a loss of enzyme activity. Indeed, under those conditions the concentration of enzymes is rate limiting. It is improbable that this reduced activity is due to cellular death, because the rate of synthesis of total nitrogen, RNA and several enzymes (Table I) is not altered under those conditions. In addition, in regenerating liver X-radiation does not lead to the release of hydrolytic enzymes from cytoplasmic granules, a phenomenon which constitutes one of the earliest manifestations of autolysis[14]. The reduced enzyme activity observed after X-radiation must therefore be due either to the formation of enzyme inhibitors, to denaturation of the enzymes or to interference with biosynthesis of the enzymes. Experiments which will be published in detail later where irradiated cytoplasm was recombined with non-irradiated cytoplasm seem to exclude the existence of inhibitors. It is doubtful that the relatively small doses of X-radiation used *in vivo* denature the enzyme. The comparison of the results obtained 6 or 24 h after X-radiation also tend to exclude such a mechanism. Indeed, in absence of significant reduction of the cell population, denaturation would (in those animals irradiated 24 or 36 h after hepatectomy), lead to a similar drop in enzyme activity whether they are sacrificed 6 or 24 h after irradiation.

The results obtained in those animals irradiated 14 h after hepatectomy and sacrificed 6 h later suggest that X-radiation interferes with the biosynthesis of the enzyme involved in the last steps of DNA biosynthesis. Indeed, under those conditions, the animals are irradiated when no enzyme activity is measurable and sacrificed when DNA synthesis is very active. While this study was in progress two abstracts appeared describing experiments where partially hepatectomized rats were irradiated before the occurrence of DNA synthesis in the regenerating liver and sacrificed 6 h later[15,16]. Although these experiments were done under different conditions and only at one concentration of enzymes, the results are in agreement with those presented here. A recent report of BOLLUM *et al.*[13], suggests that reduced incorporation of DNA precursors after X-radiation results from interference with the activity of several enzymes involved in the last step of DNA synthesis. It is not astonishing that little or no effect of X-radiation could be measured when animals irradiated 24 or 36 h after hepatectomy are sacrificed 6 h later. Indeed, under those conditions large amounts of enzymes are already present in the regenerating liver at the time of irradiation, and, therefore, significant changes in activity will become manifest only if sacrifice occurs later (24 h, for example) after irradiation.

The reduced incorporation of [^{3}H]DCMP into irradiated nuclei in presence of regenerating rat liver supernatant confirms our previous observations in homogenates[2]. This may result either from direct effect of X-radiation on DNA or from interference with the mechanism that makes nuclear DNA available as primer. The nature of that effect needs to be investigated with an enzyme preparation free of deoxyribonuclease activity.

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INACTIVATION OF ENZYME FORMATION BY ULTRAVIOLET LIGHT

I. ACTION SPECTRA AND SIZE OF THE SENSITIVE UNIT

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SUMMARY

The action spectra for loss of ability to form the inducible enzymes β-galactosidase and tryptophanase by *Escherichia coli*, and also for colony formation, resemble the absorption spectra of nucleic acids. A minimum size of the sensitive unit for enzyme formation of 300,000 molecular weight units is computed. This suggests that the sensitive unit is either high molecular weight RNA or DNA and not soluble RNA. On the basis of previously reported quantum yields, the size of the target is estimated to be somewhat larger than the minimum, perhaps of mass 700,000. This result is in fair agreement with present estimates of the size of a functional gene or of the RNA of a ribonucleoprotein particle. These data do not permit a choice between RNA and DNA as target materials.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

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INTRODUCTION

An action spectrum is the representation of the relative efficiency of radiation of different wave lengths in bringing about an effect in an irradiated system[1,2]. These action spectra are displayed as a plot of the reciprocal of the amount of light required to produce a given effect as a function of wave length. Close similarity of the action spectrum and the absorption spectrum of some component of the system is taken as evidence that the initial effect of the light is on this component[3].

In the present communication the effect of u.v. light on the formation of bacterial enzymes is considered. An effect on such a phenotypic property, virtually instantaneous and measurable immediately after irradiation, would seem susceptible of more ready interpretation than the commonly investigated effect on viability which represents a complex of many independently affected processes. Action spectra for enzyme formation have been reported for the galactozymase of yeast[4] and the lysine decarboxylase of *Bacillus cadaveris*[5]. In each case, the action spectrum was reported to resemble the absorption spectrum of nucleic acid; the results provided support for the often stated contention that nucleic acids are involved in enzyme synthesis.

The present communication extends the data to include two other enzyme forming systems, those of β-galactosidase and tryptophanase of *Escherichia coli*. The former enzyme is of particular interest because of the extensive investigations carried out on the mechanism of its induction[6]. Both enzymes can be induced in readily determined amounts in a fraction of an hour. In the present experiments, the range of wave lengths used was extended to below 240 mμ where the difference between the absorption spectra of protein and nucleic acid are most evident. The data also permit a calculation of the size of the photosensitive unit.

MATERIALS AND METHODS

The organisms used, a radiation resistant strain of *E. coli*, B/r, or *E. coli* K12 were grown aerobically by swirling at 37° in a synthetic medium, M63, containing per liter 2 g glycerol and the following salts: 13.6 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 7.0 with KOH. The bacteria were harvested in the exponential phase of growth, and were irradiated with an apparatus (made available by Dr. C. A. KNIGHT) having as its principal components an AH6 General Electric lamp operating at 500 V and a Bausch and Lomb diffraction grating to obtain the action spectrum. Details of the technique have been described elsewhere[7]. The samples were vigorously stirred during the irradiation period of from 2.5 to 30 min. The actinometry[8] necessary to measure light intensities was performed immediately following the irradiation of each sample and under the same conditions.

In the preliminary experiments a u.v. sterilizing lamp (15 W Sylvania Germicidal-A) with principal output at 2537 Å was used and intensity was determined by a u.v.-sensitive photoelectric meter.

β-galactosidase was induced with $5 \cdot 10^{-4}$ *M* isopropyl-β-D-galactoside and assayed by the *o*-nitrophenyl-β-galactoside procedure[9]. Tryptophanase was induced with $3 \cdot 10^{-3}$ *M* tryptophan and determined by indole production, as measured with Ehrlich's reagent[10]. Viability of the bacteria was measured by plating on broth-agar, using suitable precautions to avoid photoreactivation.

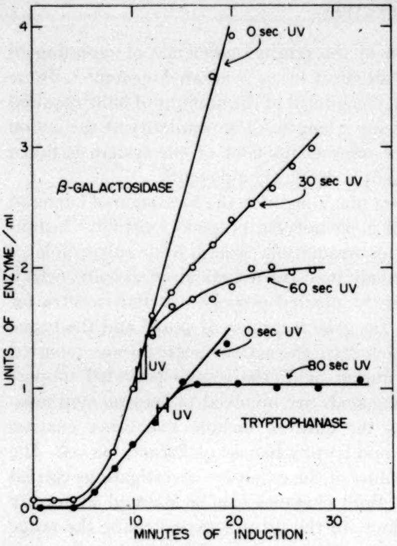

Fig. 1

Fig. 1. Kinetics of inactivation of enzyme formation. The bacteria, *E. coli* K12, were grown and induced at 37° for tryptophanase or at 24° for β-galactosidase. In either case, a portion of the culture was irradiated at an intensity of 27 ergs/mm^2/sec u.v. light (2537 Å) at the times and for the durations indicated on the Figure. Irradiation was performed in the growth medium. Aliquots for enzyme assay were taken before irradiation, and from both irradiated and unirradiated samples after irradiations. The data are plotted as enzyme activity (mμ-moles of indole or *o*-nitrophenol produced/min/ml of culture) against time of sampling.

Fig. 2. Enzyme inactivation as a function of dose. *E. coli* K12 were grown and then irradiated with 27 ergs/mm^2/sec u.v. light (2537 Å) as described under METHODS. The rate of enzyme formation in the first 40 min after induction is plotted against u.v. dose.

Fig. 3. Action spectra. Reciprocals of the doses required to reduce viability to 5 %, β-galactosidase formation to 20 %, or tryptophanase formation to 10 % of their unirradiated-control rates, divided by the comparable values obtained at 2650 Å, are plotted as a function of wave length. For comparison, an action spectrum for inactivation of aldolase and an absorption spectrum for nucleic acid are shown.

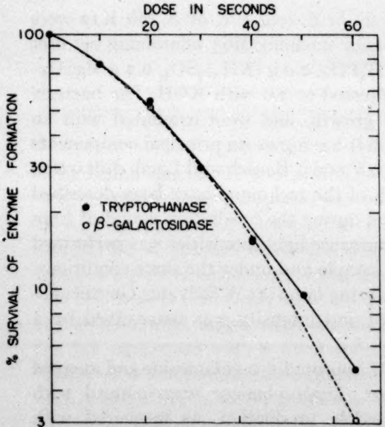

Fig 2

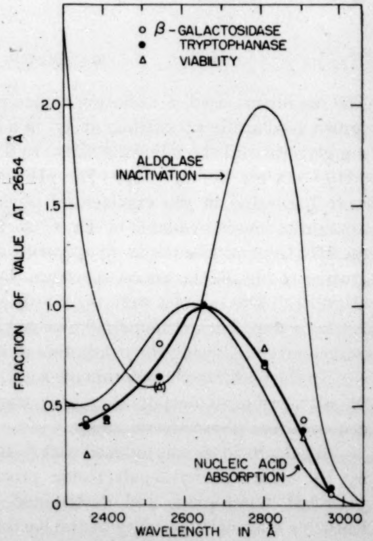

Fig 3

RESULTS

Formation of tryptophanase or β-galactosidase was arrested almost at once following irradiation (Fig. 1). This result suggests that u.v. light has a rather direct effect on enzyme formation, and that there has not been much opportunity for repair processes and secondary effects of irradiation to confuse the interpretation. No evidence for reactivation, by visible light or nutrients, of enzyme forming ability has been found under the present conditions. Other experiments showed that the reciprocity law[1] applied, since the same effect was observed if the total dose was kept constant although applied over different lengths of time (Table I). Irradiation of *E. coli* with different doses of u.v. light resulted in a nonlinear decline of ability to form the enzymes as shown in Fig. 2. Interpretation of these curves in terms of metabolism and multiplicity of targets will be reserved for a later paper.

TABLE I

EFFECT ON β-GALACTOSIDASE FORMATION OF CONSTANT U.V. DOSE GIVEN OVER VARIABLE TIMES

E. coli B/r suspended in medium lacking a carbon source at 0° were irradiated for the times and at the intensities shown. Intensity was varied by altering the lamp-to-sample distance. The samples were then induced at 37° with $5 \cdot 10^{-4}$ *M* IPTG, after addition of glycerol. Rates of β-galactosidase formation in the 40 min following irradiation are shown in the last column as % of control.

Expt.	*Dose ergs/mm^2*	*Intensity ergs/mm^2/sec*	*Irradiation time (min)*	*Enzyme formation, % remaining*
I	1270	11.2	1.9	12
I	1270	4.4	4.8	14
I	1270	1.4	15.0	11
I	2110	11.2	3.1	0.6
I	2110	4.4	8.00	0.8
I	2110	1.4	25.0	0.6
II	700	75	0.16	64
II	700	23	0.50	72
II	700	12.5	0.93	67
II	1200	75	0.27	32
II	1200	23	0.83	22
II	1200	12.5	1.57	29

To obtain the action spectra, aliquots of the bacterial suspension were irradiated for two different times at each of six wave lengths and the ability of the bacteria to produce enzymes or form colonies were compared with corresponding abilities of unirradiated samples (Table II). Whereas the ability to form β-galactosidase was well retained after storage at 0°, that for tryptophanase was lost in part. These data were plotted as in Fig. 2 to permit estimation of the quantities of light required to produce a given per cent inactivation and the reciprocal of these doses were plotted against the corresponding wave length (Fig. 3). The absorption spectra for nucleic acid[11] and for inactivation of the enzyme aldolase[12] (normalized to unity at 2654 Å) are also shown in the Figure. The data for all three properties are similar to the curve for nucleic acid absorption, and are not like that for protein inactivation.

TABLE II

INACTIVATIONS BY U.V. LIGHT

The experiment was performed as described under METHODS. The bacteria were washed and resuspended at 0° in medium lacking glycerol prior to irradiation. Aliquots were irradiated for the times indicated at approx. 12°, and then were stored at 0° until the irradiations were finished. Inductions and assays were then performed on various samples to which glycerol was added. Values at 2967 Å from a separate experiment.

Wave length Å	*O.D.*	*Photons/ml/min* × 10^{-15}	*Irradiation (min)*	*Per cent survival*		
				Viable bacteria	*β-galactosidase*	*Tryptophanase*
—	—	—	0	(100)	(100)	(100)
2350	0.167	0.81	15	43	62	27
2350	0.167	0.81	30	15	24	9.3
2399	0.148	1.19	10	24	53	21
2399	0.148	1.19	20	2.3	20	8.2
2537	0.163	1.48	10	(5)	17	7.1
2537	0.163	1.48	20	< 0.1	0.5	2.2
2654	0.163	4.03	2.5	3.6	25	5.5
2654	0.163	4.03	5	0.2	4.8	3.8
2803	0.131	3.94	2.5	5.7	33	12
2803	0.131	3.94	5	1.0	15	7.7
2894	0.104	4.10	5	8.2	33	14
2894	0.104	4.10	10	0.4	2.8	2.2
2967	0.131	8.45	6	26	97	57
2967	0.131	8.45	10	4	49	34

DISCUSSION

That an action spectrum should have the same shape as the absorption spectrum of the light-sensitive material can be seen from the following argument. It is assumed that a standard biological effect is produced by the same number of altered molecules, irrespective of the wave length of irradiation[1]. If I_0 is the intensity of incident irradiation, t is the duration of irradiation, and D is the total O.D. of the sample, the amount of light absorbed is $I_0t(1-10^{-D})$. The fraction of this light absorbed by the sensitive substance is A/D, where A is the O.D. of the sensitive substance. The amount of light absorbed by the latter times the quantum yield (ϕ, equal to inactivating events per quantum absorbed) equals the number, N, of damaged molecules:

$$N = \phi A I_0 t\,(1-10^{-D})/D.$$

As the O.D. approaches zero, $(1-10^{-D})/D$ approaches unity, and for a constant amount of biological damage the reciprocal of the dose (I_0t) becomes proportional to the O.D. of the sensitive material (A). If ϕ is assumed constant at all wave lengths, this reciprocal of the dose for the given effect should be proportional to A at all wave lengths. For comparison of action spectra with absorption spectra, one also has to make the assumption that the light absorption by the sensitive material in the cell is proportional at all wave lengths to its extinction coefficient in the free state.

For a valid comparison to be made between action spectra and absorption spectra, various experimental requirements must be fulfilled[1]. These include low O.D. of the suspension and adherence to the reciprocity law. Also it would seem desirable to extend the measurements over a wave length range that distinguishes between alternative spectra, corresponding to inactivations of nucleic acids or proteins or required for free radical formation and to use methods of measurement of the biological phenomena that are little subject to complications of secondary effects of the irradiation. Since these precautions were taken into account, the present results are in agreement with earlier results in stating that microbial viability[1] and enzyme forming ability[4,5] are inactivated by u.v. light as a result of damage to nucleic acid.

If it is assumed from the action spectrum that the u.v. sensitive unit is nucleic acid, the data permit an estimation of its size. In the region of Fig. 2, at about 10 % survival, where log of enzyme forming ability is decreasing approximately linearly with dose, an average of one effective hit per unit decreases the rate of enzyme formation by 1/e. At the maximum effective wave length, 2654 Å, $1.2 \cdot 10^{15}$ photons were absorbed per ml to bring about such a reduction of β-galactosidase formation (Table II). (A correction of 25 % for light scatter was made in computing the amount of light absorbed. The correction was estimated by comparing the O.D. of intact and sonically disrupted bacteria.) This amounts to $8 \cdot 10^6$ photons absorbed per bacterium for enzyme inactivation to 1/e (compared to $5 \cdot 10^6$/bacterium, earlier reported for a lethal effect[13]). If the sensitive unit must absorb $(1/\phi)$ photons on the average for every inactivating hit, then $(1/\phi)/1.2 \cdot 10^{15}$ equals the ratio of the photons absorbed by each sensitive unit to those absorbed by the total material in 1 ml. The ratio of light absorbance of sensitive material and nucleic acid are assumed to be proportional to their relative masses, a reasonable assumption since nucleic acid absorbs virtually all the light at this wave length. Then the sensitive unit must have a mass of $(1/\phi)/1.2 \cdot 10^{15}$ of that of the nucleic acid in one ml. There are approx. $5 \cdot 10^{-6}$ g of nucleic acid/ml, as determined[11] from the O.D. of 0.12. Therefore the size of the sensitive unit equals 2500 $(1/\phi)$ molecular weight units.

Although $(1/\phi)$ is not known for this system, a minimum value can be estimated. Uracil and cytosine nucleotides are altered by u.v. light with a $(1/\phi)$ of about 50, much more efficiently than are purine nucleotides or thymidine[14]; similar values are reported for alteration of pyrimidines in nucleic acids[15]. If, as estimated from base ratios, about 40 % of the light is absorbed by the sensitive pyrimidines in RNA, or 25 % in DNA, 120 photons would be absorbed by the nucleic acid per chemical alteration of the RNA, or 200 for DNA. Then assuming $(1/\phi) = 120$ or 200, the sensitive unit would have a mass of 300,000 or 500,000, respectively. If the former is taken as a minimum estimate, it appears that the unit is not a low molecular weight RNA such as soluble RNA. The reasonable possibilities for the identity of the sensitive material are ribonucleoprotein RNA or DNA.

The use of a value of 120 for $(1/\phi)$ is questionable in these experiments because there is no certainty that every photochemical event which modified a pyrimidine nucleotide *in vitro* must cause loss of activity of a sensitive unit in nucleic acid. An estimate of $(1/\phi)$ for biological effects places the value somewhat higher. For inactivation of infectivity of RNA of tobacco mosaic virus, as determined by the same techniques used in this study[7], $(1/\phi)$ was equal to 280, and higher values have been reported (see ref. 7). Also, an estimate of $(1/\phi)$ for transformation of optochin resistance by transforming principle gave virtually the same value[16]. By use of this figure, the sensitive unit for enzyme formation would have a mass of 700,000. The mass of the sensitive unit is reasonably similar to estimates of the size of DNA in a functional gene[16,17] or to the RNA in a ribonucleoprotein particle[18]. The data do not permit decision whether it is more likely DNA or RNA that is the sensitive substance.

ACKNOWLEDGEMENTS

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BIOSYNTHESIS OF ACID-SOLUBLE NUCLEIC ACID PRECURSORS IN ULTRA VIOLET-IRRADIATED AND CHLORAMPHENICOL-TREATED *ESCHERICHIA COLI* B

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SUMMARY

In cultures of *E. coli* B, in which both DNA and protein synthesis are permanently inhibited by u.v.-irradiation, followed immediately by the addition of chloramphenicol, very large losses of intracellular nucleic acid precursors were observed during incubation after irradiation. If the antibiotic is added 35 min after irradiation, which affects only the permanent inhibition of protein synthesis, but does not prevent the resuming of DNA synthesis after the temporary inhibition induced by u.v.-irradiation, no such losses can be observed.

The possible explanations of the above results are discussed.

INTRODUCTION

It has been demonstrated (HAROLD AND ZIPORIN[2]; DRAKULIĆ AND ERRERA[1]) that the addition of chloramphenicol immediately after u.v.-irradiation permanently inhibits DNA synthesis in *Escherichia coli* B.

In view of the fact that the temporary inhibition of DNA synthesis which takes place in *E. coli* B by u.v.-irradiation alone is accompanied by an accumulation of DNA derivatives (KANAZIR AND ERRERA[3]), we were interested to know whether we could observe a similar phenomenon in u.v.-irradiated and chloramphenicol-treated cells.

MATERIALS AND METHODS

E. coli B was maintained and cultivated as described earlier (DRAKULIĆ AND ERRERA[1]).

Absorbancy measurements were made with a Beckman model DU spectrophotometer, in 10 mm depth cuvettes at a wavelength of 660 mμ.

Chemical fractionations of the bacteria were carried out by the somewhat modified OGUR-ROSEN procedure (OGUR AND ROSEN[5]). 10–20 ml of bacterial suspension were centrifuged, washed with cold physiological saline solution, and delipidated twice with 5 ml ethanol–ether (3:1) by stirring with glass rods in an ice bath for 20 min each time.

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

The acid-soluble nucleic acid precursors were extracted with 2 ml of 2 % perchloric acid at 4° by stirring with glass rods during 20 min. After centrifugation the precipitate was washed once more with 2 % perchloric acid, and the extract and the wash were pooled. RNA was extracted with 10 % perchloric acid in the refrigerator during 30–40 h.

The nucleic acid precursors and RNA were determined by their absorption maxima at 260 mμ, as measured with a Beckman model DU spectrophotometer, in 10-mm depth quartz cuvettes. The amount of RNA was calculated on the basis of the fact that an extinction of 1.00 corresponds to 35,5 μg. In some experiments RNA was also determined by the orcinol reaction (MEJBAUM[4]).

The u.v.-spectra of the media were also determined with a Beckman model DU spectrophotometer, in 10-mm depth quartz cuvettes.

Irradiation was performed on 55-ml aliquots of bacterial suspension in 13,5-cm diameter Petri dishes stirred with the hand, at a distance of 100 cm from a Mazda low-pressure mercury lamp, giving approx. 540 ergs/mm^2/min of 253,7 mμ u.v.-light.

EXPERIMENTS AND RESULTS

A log phase culture of *E. coli* B at an absorbancy of 0.100–0.200 was divided into two parts, one of which was irradiated with a total dose of 900 ergs u.v./mm^2, the other being kept as a control.

We performed two types of experiments with these cultures.

Experiment I

Immediately after irradiation the control and the irradiated cultures were divided into two parts. To one part of each, a solution of chloramphenicol was added to a final concentration of 10 μg/ml.

Thus we worked, as we did in the experiments described in an earlier paper (DRAKULIĆ AND ERRERA[1]), with four suspensions:

(a) control culture
(b) irradiated culture
(c) control culture plus chloramphenicol
(d) irradiated culture plus chloramphenicol

These cultures were reincubated after irradiation at 37° with aeration by bubbling air through the suspensions. Samples for absorbancy measurements and chemical analysis were taken at the time intervals, shown in Fig. 1, which shows the synthesis of nucleic acid precursors, and in Fig. 2, which shows growth and RNA synthesis.

The synthesis of nucleic acid precursors in the control culture was slowed down less than the growth was. In the irradiated culture the addition of chloramphenicol immediately after irradiation did not change the synthetic pattern of the precursors for the first 40 min; but after that period a large diminution of the u.v.-absorption in the 2 % perchloric acid extract for some 3 h or more was observed.

Fig. 2 shows that the addition of chloramphenicol slows down RNA synthesis considerably in both the control and the irradiated suspensions after some 40 min. However, a permanent inhibition of RNA synthesis was never observed in our experiments.

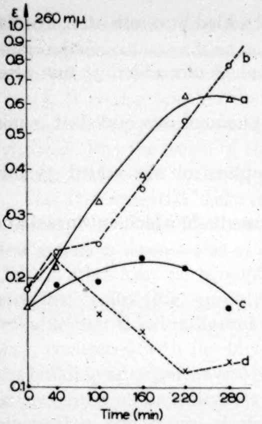

Fig. 1. Synthesis of acid-soluble nucleic acid precursors in four cultures of *E. coli* B: a = control; b = irradiated; c = control plus chloramphenicol added at 0 min; d = irradiated plus chloramphenicol added at 0 min.

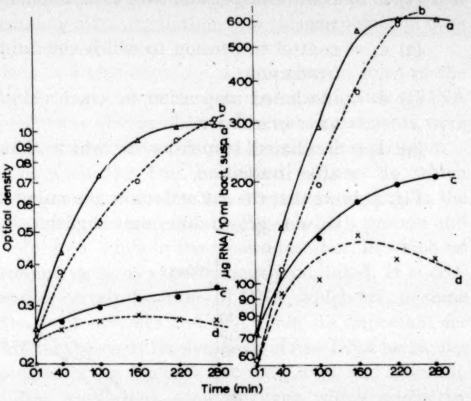

Fig. 2. Growth (left) and RNA synthesis (right) in four cultures of *E. coli* B: a = control; b = irradiated; c = control plus chloramphenicol added at 0 min; d = irradiated plus chloramphenicol added at 0 min.

Experiment II

This type of experiment was designed to find out whether the addition of chloramphenicol 35 min after irradiation is also accompanied by a loss of intracellular nucleic acid precursors.

As Fig. 3 shows, no degradation of the acid-soluble precursors was observed under these conditions in the control culture (curve c_1), or in the irradiated culture (curve d_1).

To find out whether the loss of u.v.-absorption at 260 mμ in curve d of Fig. 1 is due to leakage of the precursors through the cellular membrane, we measured the u.v.-spectra of the media in which the following cell suspensions were grown:

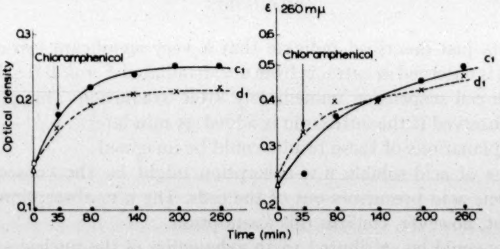

Fig. 3. Growth (left) and the synthesis of acid-soluble nucleic acid precursors (right) in a control culture (c_1) and an irradiated culture (d_1) of *E. coli* B. To both cultures chloramphenicol was added 35 min after irradiation.

(1) c = control suspension with chloramphenicol added at 0 min after irradiation.
(2) c_1 = control suspension to which chloramphenicol was added 35 min after irradiation.
(3) d = irradiated suspension to which chloramphenicol was added at 0 min after irradiation.
(4) d_1 = irradiated suspension to which chloramphenicol was added 35 min after irradiation.

Fig. 4 shows that the extinctions at 260 mμ of the media in which cultures d and d_1 were grown differ only slightly.

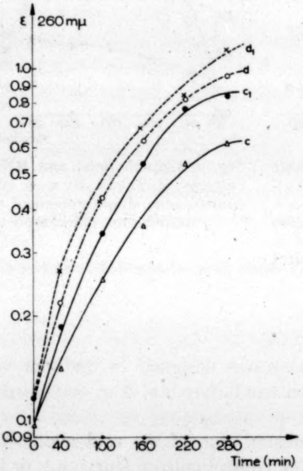

Fig. 4. Extinction at 260 mμ of the media in which four cultures of *E. coli* B have been grown: c = control plus chloramphenicol added at 0 min; d = irradiated plus chloramphenicol added at 0 min; c_1 = control plus chloramphenicol added at 35 min; d_1 = irradiated plus chloramphenicol added at 35 min.

DISCUSSION

The experiments just described indicate that a very significant loss of acid-soluble u.v.-absorption is observed in extracts from u.v.-irradiated *E. coli* B if chloramphenicol is added to the cell suspension immediately after irradiation. This phenomenon is, however, not observed if the antibiotic is added 35 min later.

Several explanations of these results could be imagined:

(1) The loss of acid-soluble u.v.-absorption might be the consequence of the leakage of nucleic acid precursors out of the cells. The u.v.-absorption of the media (Fig. 4) does not, however, confirm this assumption.

(2) The loss could be attributed to an exhaustion of the nucleic acid precursors pool, because RNA synthesis seems to be slowed down less by the addition of chloramphenicol (Fig. 2) to the irradiated cells than the synthesis of the precursors (Fig. 1).

But this assumption does not explain why the phenomenon was observed only when chloramphenicol was added immediately after irradiation, and this phenomenon does not happen if the antibiotic is added 35 min later.

(3) It is also possible that there is a feed-back, *i.e.* a repression of some of the purine, and/or pyrimidine-building enzymes, due to the permanent inhibition of DNA synthesis. But the losses of the precursors always observed in our experiments were so large that we do not think that they could be explained by enzyme repression.

(4) The essential difference between the two irradiated cultures to which chloramphenicol is added immediately or 35 min after u.v.-treatment, is that, in the first instance, when a loss of acid-soluble material occurs (Fig. 1), both protein and DNA synthesis are permanently inhibited, while in the second instance, in which no such loss occurs (Fig. 3), only protein synthesis is permanently inhibited. It is conceivable that u.v.-irradiation damages certain fractions of DNA, which are, in some way, connected with the biosynthesis of enzymes and that these are important for the building up of purine and pyrimidine bases. If the synthesis of new DNA molecules is prevented as happens, for example, when bacteria are irradiated and chloramphenicol is added immediately after irradiation, some enzymes which synthetize precursor molecules might be used up after a certain period of time, and might not be reformed. If the catabolism of the bases is affected less under these conditions, the catabolic–anabolic equilibrium would be disturbed, and a degradation of the precursors would be observed as the consequence of the disequilibrium.

If this assumption were true, it would mean that the cells contain only very limited amounts of some of the enzymes necessary for the synthesis of the nucleic acid precursors. These enzymes could represent "pace-makers", limiting the metabolic activities of normal cells.

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NUCLEAR-CYTOPLASMIC RELATIONSHIPS IN HUMAN CELLS IN TISSUE CULTURE

IV. A STUDY OF SOME ASPECTS OF NUCLEIC ACID AND PROTEIN METABOLISM IN ENUCLEATE CELLS

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SUMMARY

Enucleate pieces of cytoplasm were prepared from human amnion cells in tissue culture by a method of microsurgery. Vital activities of the fragments were retained for 10–30 h. One of several amino acids or RNA precursors labeled with ^{14}C was added to the culture medium just before or just after preparation of a group of enucleate cytoplasmic fragments. When about half the fragments had "died", the surviving enucleates were fixed and examined by autoradiography for evidence of incorporation of the labeled compound. Amino acids were incorporated as actively by enucleate cytoplasm as by cytoplasm of complete cells. This incorporation could be blocked by excess label-free L-isomers of the corresponding labeled amino acid but not by the label-free D-isomer. RNA precursors were not incorporated by enucleate cytoplasmic fragments in detectable amount, although neighboring intact cells incorporated the precursors actively into cytoplasm.

The conclusions reached are (a) that protein synthesis continues in enucleate cytoplasm of mammalian cells, but (b) that RNA synthesis stops or is so slight as to escape detection by these methods. These data add to the evidence that cytoplasmic RNA is derived chiefly from the nucleus.

The labeled precursors used were: [^{14}C]adenine, [^{14}C]uridine, L-[^{14}C]glutamic acid, L-[^{14}C]leucine, L-[^{14}C]phenylalanine and DL-[^{14}C]tryptophan; [8-^{14}C] adenylic acid was also used.

INTRODUCTION

The importance and necessity of studying the metabolism of enucleate cell fragments have been emphasized on numerous occasions[1, 2]. We would like briefly to reiterate the importance of determining conclusively whether a cell without a nucleus is capable of RNA synthesis.

Abbreviation: RNA, ribonucleic acid.

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There is abundant cytological evidence[3-6] that a substantial fraction of cytoplasmic RNA probably is of nuclear origin. This suggests that RNA (which is concerned with the synthesis of cytoplasmic protein) may be the agent for the transmission of genetic information from the chromosome. If this hypothesis is correct, one would expect no independent synthesis of RNA in the cytoplasm. The most conclusive approach presently available for establishing whether this is the case is to study the metabolism of RNA in enucleate cells. If it should be shown that RNA synthesis is possible in the absence of a nucleus, it would then be necessary seriously to reconsider the nature of the biological role of RNA.

In earlier studies on the metabolism of enucleate cells of lower form, some doubt as to the importance or validity of the observed results arose because the cells obviously manifested overt abnormality as an immediate result of enucleation. Thus, in the case of amoeba[1], locomotion either ceases at once or becomes decidedly abnormal. In sea urchin and amphibian eggs, cell division, if it proceeds at all, is also decidedly abnormal in the absence of a nucleus[7]. In the case of human tissue culture cells, however, the removal of the nucleus appears to have little immediate effect on the behavior of the cell[8]. For this reason, a study of the ability of enucleate mammalian tissue culture cells to incorporate amino acids and nucleic acid precursors was felt to hold more promise for an understanding of some aspects of the relative role of nucleus and cytoplasm. Similar studies had been made earlier with the so-called more "primitive" forms, and we have executed essentially identical experiments.

MATERIALS AND METHODS

The cells used throughout the course of these experiments were human amnion cells established in culture[9]. The culture methods, equipment and techniques employed to enucleate the cells were similar to those described earlier[8]. Fixation of cells, enzyme digestions and autoradiography have been described previously[6].

The labeled precursors used in these experiments were: [^{14}C]adenine, Isotopes Specialties Co., specific activity, 3.3 mC/mmole; [8-^{14}C]adenylic acid, Schwarz Laboratories Inc., specific activity, 1.64 μC/mg; [^{14}C]uridine, Schwarz laboratories Inc., specific activity, 0.52 μC/mg; L-[^{14}C]glutamic acid, Nuclear-Chicago Corp., specific activity, 4.67 mC/mmole; L-[^{14}C]phenylalanine, Nuclear-Chicago Corp., specific activity, 11.35 mC/mmole; DL-[^{14}C]tryptophan, Nuclear-Chicago Corp., specific activity, 4.67 mC/mmole; L-[^{14}C]leucine Nuclear-Chicago Corp., specific activity, 7.95 mC/mmole.

EXPERIMENTAL PROCEDURE

The cell-cutting operation[8], which was the method employed here for obtaining enucleate cells, required 1–2 h per chamber during which time as many as 21 enucleate fragments were produced. Labeled precursor was added either before beginning the operation or after it was completed.

When label was added after micrurgy, the medium was not covered with oil before the operation, but was covered after the labeled medium was added.

Approx. 10–20 enucleates were prepared in one operating chamber. Observations were made every 2.5 h to determine the proportion of surviving enucleates, using criteria described previously[8]. An attempt was made to fix the cells at a time when approximately one-half the enucleates had died. This was not always possible, but in all cases some enucleates had died before fixation was effected. An attempt was made to adjust the length of the period the cells were under autoradiographic film according to the length of exposure to the radioactive precursor.

The controls, with which the enucleates were compared, were nearby normal nucleate cells in the same chamber. A subjective estimate of the relative radioactivity was possible by comparing autoradiographs of the enucleates and of the intact cells.

RESULTS

Incorporation of RNA precursors

A series of $[^{14}C]$ RNA precursors (Table I) was administered to enucleated cells, but significant incorporation was not detectable over background. In all cases, the nearby normal nucleate cells showed considerable incorporation. It is clear that if the enucleates are incorporating any label, the extent of the incorporation must be considerably less than 1 % of that in nucleate cells[§].

TABLE I

SUMMARY OF EXPERIMENTS IN WHICH LABELED RNA PRECURSORS WERE ADMINISTERED TO ENUCLEATE AMNION CELLS

Experiment No.	*Precursor*	*Activity in medium (μC/ml)*	*Approx. time between operation and fixation (h)*	*No. of living enucleates at time of fixation over No. of living enucleates at start*
1*	$[^{14}C]$adenine	0.5	22	3/10
2a	$[^{14}C]$adenine	0.5	25	6/14
2b*	$[^{14}C]$adenine	0.5	21	7/13
3a	$[^{14}C]$adenine	2.0	25	13/16
3b*	$[^{14}C]$adenine	2.0	20	9/14
4a	$[^{14}C]$adenine	2.0	26	13/18
4b	$[^{14}C]$adenine	2.0	21	7/17
5a	$[^{14}C]$adenine	2.0	25	6/9
5b*	$[^{14}C]$adenine	2.0	23	11/13
6a*	$[^{14}C]$adenine	2.0	22	7/18
6b	$[^{14}C]$adenine	2.0	22	9/19
7a	$[^{14}C]$adenine	2.0	24	6/21
7b	$[^{14}C]$adenine	2.0	24	8/19
8a	$[8\text{-}^{14}C]$adenylic acid	1.0	29	6/14
8b	$[8\text{-}^{14}C]$adenylic acid	1.0	26	2/7
9a	$[8\text{-}^{14}C]$adenylic acid	1.0	30	13/18
9b	$[8\text{-}^{14}C]$adenylic acid	1.0	29	8/14
10a	$[8\text{-}^{14}C]$adenylic acid	1.0	24	3/12
10b	$[8\text{-}^{14}C]$adenylic acid	1.0	27	6/11
11a	$[^{14}C]$uridine	1.0	24	5/14
11b	$[^{14}C]$uridine	1.0	24	4/6
12a	$[^{14}C]$uridine	1.0	24	7/13
12b	$[^{14}C]$uridine	1.0	24	4/13

* Experiments designated thus were labeled after enucleation.

[§] $[^3H]$cytidine was employed in similar experiments. Variably high background and evidence that nonspecific adsorption may give autoradiographs over areas where true incorporation has not occurred indicate need for further study. Preliminary interpretations are, however, not incompatible with those from ^{14}C precursors.

It should be noted that in many instances the cells were in the presence of label for as long as 1–2 h before all the enucleation operations in one chamber were completed. As a result the label was available for the enucleate metabolic pool, since the precursor was able to penetrate the cell membrane prior to enucleation. Thus, any possible permeability barriers resulting from the absence of the nucleus would be circumvented. Furthermore, our earlier studies[8] indicated that the enucleate cell does retain the ability to take up substances from the environment by pinocytosis.

Incorporation of protein precursors

All 4 labeled amino acids (L-[^{14}C]glutamic acid, DL-[^{14}C]tryptophan, L-[^{14}C]leucine and L-[^{14}C]phenylalanine) administered to enucleate cells, at concentrations of 1–2 μC/ml, were incorporated in significant amounts. In fact, incorporation into enucleate cells appeared to be of the same order of magnitude as the incorporation into the non-nuclear cytoplasmic areas of neighboring normal cells.

Incorporation of labeled tryptophan and labeled phenylalanine into enucleate cells in the presence of excess amounts of either the unlabeled D-isomer or the unlabeled L-isomer of the labeled amino acid* was studied in greater detail. With the unlabeled L-isomer in the medium, incorporation of the label was greatly reduced in the enucleate cell fragments. (Labeling in normal cells was, of course, also greatly inhibited.) On the other hand, with the unlabeled D-isomer there was essentially no effect on the amount of incorporation. These results clearly suggest that the labeling is truly physiological in nature and that it probably is related to protein synthesis.

DISCUSSION

In view of the fact that enucleate amnion cells retain all normal visible dynamic activity for an average of about 20 h and that they are apparently capable of incorporating labeled amino acids into protein in a normal fashion, the observation that such cell fragments cannot incorporate a variety of labeled RNA precursors leads to the conclusion that no independent RNA synthesis takes place in the absence of the nucleus under otherwise normal conditions. Moreover, these experiments may be taken to mean that all the RNA of the cell is of nuclear origin. They are not conclusive experiments of course, since it is possible, for example, to imagine that the nucleus furnishes to the cytoplasm a labile cofactor which is necessary for the synthetic processes involved in independent cytoplasmic synthesis of RNA.

Our results are in excellent agreement with recent work of PRESCOTT[10], who studied the incorporation of several radioactive RNA precursors in enucleate cells of *Acanthamoeba* grown in sterile culture. PRESCOTT was unable to detect any incorporation of these precursors into enucleate cells but readily demonstrated incorporation into nucleate parts. In discussing the earlier conflicting results dealing with this problem, he concludes that a good deal of the disagreement was due to lack of equivalent control of contaminating organisms. (PRESCOTT's paper[10] should be consulted for a review of the issues.) In our experiments, as in PRESCOTT's, the cells were maintained under aseptic conditions.

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* Carrier phenylalanine was present at 0.2 mg/ml, an excess of 13.3 times the concentration of labeled precursor. Carrier tryptophan was present at 0.2 mg/ml, an excess of 8.7 times the concentration of labeled precursor.

It thus appears, on the basis of the most careful experiments, that the nucleus must be present in order for a cell to synthesize RNA.

Whether the cytoplasm with a nucleus present is capable of independent RNA synthesis is still unestablished. It is our belief that only the recent work of HARRIS[11] can be taken as support for the view that cytoplasm is capable of such independent synthesis—without any direct intervention of the nucleus. On the basis of his autoradiographic studies of incorporation of labeled precursors into tissue culture cells he believes that, although the same precursors are used to manufacture both nuclear RNA and cytoplasmic RNA, there is a separate pool of intermediates for each and that it takes the label longer to move through the cytoplasmic RNA intermediate pool. His conclusion is based on the fact that, although after a brief exposure of cells to radioactive precursors all the acid-insoluble label is in nuclear RNA, the later appearance of label in cytoplasmic RNA occurs at a rate and a magnitude incompatible with the view that nuclear RNA is a precursor of cytoplasmic RNA. He believes that the loss of label from the nucleus is due to independent turnover and that the labeling in the cytoplasm comes from an acid-soluble intermediate pool, which ordinarily is undetected in this type of study due to the methods of cell fixation employed.

WOODS[12] has carried out very similar experiments with plant root cells and his data are comparable to HARRIS'—or perhaps even more dramatic in the difference observed between the initial amount of label in the nucleus and the ultimate amount of label found in the cytoplasm. While WOODS also believes that an acid-soluble pool of label (remaining after removal of the administered label from the external medium) is responsible for the marked increase in the degree of labeled RNA in the cytoplasm, he is of the opinion that the components of the acid-soluble pool are nevertheless going through the RNA-synthesizing machinery of the nucleus.

Apparently neither point of view can be firmly established on the evidence now available. The resolution of this problem will not be simple. Nevertheless, experiments are being designed that we hope will unequivocably settle the issue one way or the other.

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THE NUCLEIC ACID METABOLISM OF ANIMAL CELLS *IN VITRO*

III. FACTORS INFLUENCING NUCLEOTIDE BIOSYNTHESIS

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SUMMARY

In the Ehrlich ascites carcinoma of the mouse the rate of purine biosynthesis *in vitro* under aerobic conditions is greatly increased if the cells are supplied with glucose or uridine, although neither compound appears to increase the energy available to the cells. Glucose and nucleosides also increase the rate at which the cells take up adenine and uracil from the medium and convert them to nucleotides. Glucose has no effect on uptake of glycine and phenylalanine under anaerobic conditions, although it increases phenylalanine uptake when the cells are incubated under nitrogen. The L5178Y mouse ascites lymphoma differs from the Ehrlich ascites carcinoma in having a much higher content of glucose *in vivo*, a lower capacity for glucose uptake *in vitro*, and a higher capacity for purine nucleotide biosynthesis *in vitro*. In rabbit bone marrow, purine nucleotide biosynthesis *in vitro* is slow relative to nucleic acid biosynthesis, is not affected by the presence of glucose, and is only slightly increased by a mixture of glucose, glutamine, aspartic acid and glycine. It differs from the corresponding process in the ascites carcinoma in continuing at a steady rate for a longer period, and in being more sensitive to the inhibitory effects of azaserine. Chick embryo brain and skeletal muscle also synthesize purine nucleotides relatively slowly *in vitro*. In the latter, but not the former, the synthesis is accelerated by a mixture of glucose and glutamine.

INTRODUCTION

It has previously been shown that *in vivo* [^{14}C]formate is extensively incorporated into the nucleic acid purines and DNA thymine of rapidly-growing animal tissues. *In vitro*, however, many such tissues show only a limited incorporation of [^{14}C]formate into nucleic acid purines, although incorporation into DNA thymine remains high[1-5]. Experiments with "L" strain mouse fibroblasts grown in tissue culture have suggested

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Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate, DPNH, reduced diphosphopyridine nucleotide; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

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that this type of incorporation *in vitro* is due to a low rate of purine nucleotide biosynthesis, so that the purine nucleotides required for the formation of new nucleic acid molecules are derived mainly from the existing unlabeled purine nucleotides of the cell, rather than from the products of current synthesis[5]. In the Ehrlich ascites carcinoma there is evidence that the low rate of purine biosynthesis found *in vitro* is due to the absence of the glucose supply which the cells normally receive *in vivo*[4,6]. Much higher rates of purine nucleotide biosynthesis can be obtained *in vitro* by addition of glucose to the incubation medium. However, in other rapidly-growing tissues purine nucleotide biosynthesis does not appear to be dependent on glucose supply. Thus the low rate of synthesis in rabbit bone marrow *in vitro* is not increased by addition of glucose. The experiments described in the present paper were undertaken to obtain more information about the factors influencing the rate of purine nucleotide biosynthesis in rapidly-growing tissues and, in particular (a) to elucidate the mechanism by which glucose affects purine nucleotide biosynthesis in the Ehrlich ascites carcinoma, (b) to determine whether the corresponding process in bone marrow is similarly dependent on an external supply of some precursor analogous to glucose, (c) to investigate the extent to which the behaviour of bone marrow or the Ehrlich ascites carcinoma is typical of other tissues whose capacity for purine nucleotide biosynthesis *in vitro* is limited.

EXPERIMENTAL

Biological material

The Ehrlich ascites carcinoma and the L5178Y ascites lymphoma[7] were kindly supplied by Dr. G. Popjak of Hammersmith Hospital, London and Dr. G. A. Fischer of Yale University, New Haven, Conn., respectively. The Ehrlich tumour was maintained in mice of the departmental colony and the lymphoma in DBA/2 F1 hybrid mice. C3H mice bearing a transplantable mammary adenocarcinoma were kindly provided by Dr. B. D. Pullinger and Dr. A. Head of the Royal Beatson Memorial Hospital, Glasgow. This tumour originated as a spontaneous carcinoma in an ex-breeding female of the C3Hf/He strain[8,9]. For experiments on normal mouse tissues, animals from the departmental colony were used. Rat tissues were obtained from albinos of the departmental colony, weighing between 200 and 250 g. The rabbits employed were young albinos weighing approximately 1500 g. Chick embryos were obtained from eggs supplied by the Poultry Research Centre, Edinburgh.

Experimental procedures

In experiments with ascites tumours *in vitro*, tumour material from several animals was pooled, isotope was added, and 5-ml portions were dispensed into 25-ml conical flasks containing the appropriate additions. These were then incubated at 37° and simultaneously shaken at a rate of 60 to 80 oscillations/min. Bone marrow was similarly incubated as a suspension of cells[10] in Krebs–Ringer bicarbonate buffer. Other solid tissues were incubated in Krebs–Ringer bicarbonate buffer in the form of slices approximately 0.3 mm thick prepared in a McIlwain and Buddle tissue slicer[11]. Each flask contained approx. 0.5 to 1.0 g tissue in 5 to 10 ml buffer. All incubations, unless otherwise stated, were for a period of 4 h.

Analytical methods

The analytical methods for measuring incorporation of [^{14}C]formate and other labeled precursors into nucleic acids and acid-soluble nucleotides were those previously described[3]. In experiments to determine the rate at which ascites tumour cells take up various labeled compounds from the suspension medium, the flasks and their contents were chilled in ice at the end of the incubation period. The cells were then centrifuged down, washed three times with Krebs–Ringer bicarbonate and finally disrupted by suspension in distilled water.

Chromatography

The chromatographic methods used to separate the free bases obtained on hydrolysis of nucleic acids and acid-soluble nucleotides were those previously described[3]. In experiments on uptake of [2-^{14}C]uracil the acid-soluble fraction of the cells was concentrated by lyophilization, applied to paper and chromatographed in butanol–water[12] to separate free bases and nucleosides from nucleotides. In experiments on uptake of [8-^{14}C]adenine a similar procedure was followed except that two-dimensional chromatography was used with distilled water as solvent in the first dimension and butanol–acetic acid[13] in the second.

Methods of estimation

Glucose was determined by the glucose oxidase/peroxidase method (Boehringer). Pyruvate was estimated by allowing it to react with DPNH in presence of lactic dehydrogenase and observing the resultant change in optical density at 340 mμ (Boehringer). Lactate was determined by the method of HULLIN AND NOBLE[14]. ATP was estimated by allowing it to react with 3-phosphoglyceric acid in presence of phosphoglycerokinase and measuring the 1,3-diphosphoglyceric acid produced by observing the change in O.D. at 340 mμ in presence of DPNH and phosphoglyceraldehyde dehydrogenase (Boehringer). ADP was estimated by allowing it to react with phosphoenolpyruvate in presence of pyruvate kinase and measuring the pyruvate liberated (Boehringer). AMP was estimated by converting it to ADP in presence of ATP and myokinase and estimating the ADP as described above (Boehringer). In all the Boehringer methods reagents supplied by C. F. Boehringer & Soehne, GmbH., Mannheim, Germany, were used.

RESULTS

The Ehrlich ascites carcinoma

The pattern of incorporation of [^{14}C]formate into the nucleic acid bases of the Ehrlich ascites carcinoma *in vitro* is shown in Table I. In absence of any additions, incorporation into DNA adenine is disproportionately low compared to incorporation into DNA thymine. Increased incorporation into nucleic acid adenine can be obtained by addition of glucose or uridine. This appears to be a specific effect: incorporation into DNA thymine is not affected to a comparable degree. Uracil, deoxyuridine and deoxycytidine have no effect[6]. To determine whether these effects are due to glucose and uridine acting as sources of energy, the levels of AMP, ADP and ATP in the cells were estimated (Table II). *In vivo*, the cells have a high content of ATP and relatively little AMP or ADP. Incubation *in vitro* under aerobic conditions does not materially

TABLE I

INCORPORATION OF [^{14}C]FORMATE INTO THE ACID-SOLUBLE AND NUCLEIC ACID BASES OF THE EHRLICH ASCITES CARCINOMA AND NORMAL RABBIT BONE MARROW *in vitro*

Initial isotope concentration 5 μC/ml.

Experiment No.	*Tissue*	*Additions to incubation medium*	*Special conditions*	*Specific activity (counts/min/μmole)*			
				Acid-soluble adenine	*RNA adenine*	*DNA adenine*	*DNA thymine*
1	Ehrlich ascites carcinoma	Nil	Nil	—	1,460	230	9,150
		Glucose (5.0 m*M*)	Nil	—	15,000	2,740	9,000
		Uridine (0.5 m*M*)	Nil	—	10,200	2,100	12,100
2	Ehrlich ascites carcinoma	Nil	Nil	16,680	1,130	480	9,240
		Glucose (5.0 m*M*) + glutamine (1.0 m*M*)	Nil	63,000	6,600	2,110	14,900
		Nil	Cells incubated 30 min before isotope added	10,500	590	200	9,420
		Glucose (5.0 m*M*) + glutamine (1.0 m*M*)	Cells incubated 30 min before isotope added	5,100	370	60	11,040
3	Bone marrow	Nil	Nil	—	1,130	260	6,250
		Glucose (5.0 m*M*)	Nil	—	1,010	250	6,280
		Uridine (0.5 m*M*)	Nil	—	1,910	450	9,780
		Cytidine (0.5 m*M*)	Nil	—	1,640	280	6,380
4	Bone marrow	Nil	Cells washed 3 times with buffer before incubation	4,180	4,040	460	8,150
		Glucose (5.0 m*M*), glutamine (0.5 m*M*), aspartic acid (0.5 m*M*), glycine (0.5 m*M*)	Cells washed 3 times with buffer before incubation	16,700	19,100	1,930	14,600
5	Bone marrow	Nil	Nil	88,800	7,950	790	11,100
		Nil	Cells incubated 15 min before isotope added	83,800	6,520	590	9,250
		Nil	Cells incubated 30 min before isotope added	86,000	6,470	610	9,440
		Nil	Cells incubated 60 min before isotope added	83,100	5,670	420	6,700
6	Bone marrow	Nil	Nil	—	4,190	2,720	51,500
		Nil	Cells suspended in blood serum instead of buffer	—	8,530	7,130	118,400

TABLE II

EFFECT OF INCUBATION *in vitro* ON AMP, ADP AND ATP CONTENT OF ASCITES TUMOURS

Tumour	*Additions*	*Time (min)*	*AMP µmole/ml*	*ADP µmole/ml*	*ATP µmole/ml*
Ehrlich ascites	Nil	0	0.101	0.168	0.545
Carcinoma	Nil	60	0.049	0.125	0.504
(in ascitic serum)	Glucose (5.0 m*M*)	60	0.088	0.188	0.388
L5178Y ascites	Nil	0	0.028	0.054	0.097
Lymphoma	Nil	60	0.021	0.028	0.297
(in ascitic serum)	Glucose (5.0 m*M*)	60	0.028	0.022	0.212
Ehrlich ascites	Nil	0	0.051	0.073	0.681
Carcinoma	Nil	60	0.033	0.046	0.551
(in buffer)	Glucose (5.0 m*M*)	60	0.028	0.031	0.596

alter this situation, except insofar as the presence of glucose or uridine in the incubation medium tends to depress the ATP level slightly (Tables II and III). Similar results are obtained if the cells are incubated, not in their own serum, but in Krebs–Ringer bicarbonate buffer (Table II). The situation during anaerobic incubation is quite different: within 15 min there is a sharp fall in ATP content, though this can be delayed by addition of glucose (Table III). Addition of uridine has no effect. The source of the energy which the cells obtain when incubated aerobically in the presence of glucose may be found, at least in part, in the oxidation of lactate and pyruvate, which are present in high concentration in the tumour *in vivo* and which are measurably diminished during aerobic incubation *in vitro* (Table IV).

It was previously shown that the incorporation of [8-^{14}C]adenine and [6-^{14}C]orotic acid into the nucleic acids of Ehrlich ascites tumour cells incubated *in vitro* under aerobic conditions is substantially increased by addition of glucose[4]. Table V shows that under such conditions glucose also enhances uptake of both these precursors and of [2-^{14}C]uracil from the ascitic serum into the cells. In contrast, uptake of [2-^{14}C]-glycine and [2-^{14}C]phenylalanine during aerobic incubation is unaffected by addition

TABLE III

EFFECT OF GLUCOSE, URIDINE AND IODOACETATE ON THE ATP CONTENT OF THE EHRLICH ASCITES CARCINOMA *in vitro*

Gas phase	*Time of incubation (min)*	*Additions*	*ATP content (µmole/ml)*
Air	0	Nil	0.414
	45	Nil	0.526
	45	Glucose (20 m*M*)	0.396
	45	Uridine (0.5 m*M*)	0.396
	45	Iodoacetate (1.0 m*M*)	0.084
N_2	0	Nil	0.368
	15	Nil	0.086
	40	Nil	0.073
	15	Glucose (10 m*M*)	0.465
	40	Glucose (10 m*M*)	0.110
	15	Uridine (0.5 m*M*)	0.060
	40	Uridine (0.5 m*M*)	0.048

of glucose. Under anaerobic conditions uptake of [2-^{14}C]phenylalanine is depressed, but can be restored by addition of glucose (Table V). Uptake of [2-^{14}C]uracil under aerobic conditions is increased by addition of adenosine; and, conversely, uptake of [8-^{14}C]adenine is increased by addition of uridine (Table V). When [8-^{14}C]adenine or [^{14}C]uracil is taken up by ascites cells *in vitro*, part of the radioactivity in the cells is found in the form of nucleotides and part as free base (Table VI). If either glucose

TABLE IV

EFFECT OF INCUBATION *in vitro* ON LACTATE AND PYRUVATE CONTENT OF THE EHRLICH ASCITES CARCINOMA

Time (min)	*Gas phase*	*µmoles/ml lactate*	*Tumour pyruvate*
0	—	10.9	0.23
40	Air	10.4	0.24
120	Air	9.0	0.08
40	O_2	10.2	—
120	O_2	9.9	—

TABLE V

THE EFFECT OF GLUCOSE, RIBOSE AND NUCLEOSIDES ON UPTAKE OF LABELED AMINO ACIDS, PURINES AND PYRIMIDINES BY EHRLICH ASCITES CARCINOMA CELLS *in vitro*

	Gas phase	*Control*	*Percentage of added radioactivity taken up by cells*				
			+ Glucose (5 mM)	*+ Glucose (20 mM)*	*Uridine (0.5 mM)*	*Adenosine (0.5 mM)*	*Ribose (20 mM)*
0.025 m*M* [8-^{14}C]adenine	Air	47	62	70	73	78	—
0.043 m*M* [2-^{14}C]uracil	Air	4	16	25	—	30	3
0.043 m*M* [6-^{14}C]orotic acid	Air	4	6	9	—	—	—
0.053 m*M* [2-^{14}C]glycine	Air	46	48	46	—	—	—
0.020 m*M* [2-^{14}C]phenylalanine	Air	40	43	44	—	—	—
Ditto	N_2	28	33	45	—	—	—

TABLE VI

DISTRIBUTION OF RADIOACTIVITY IN ACID-SOLUBLE FRACTION OF EHRLICH ASCITES CARCINOMA CELLS INCUBATED AEROBICALLY *in vitro*

Incubation in presence of 0.025 m*M* [8-^{14}C]adenine or 0.043 m*M* [2-^{14}C]uracil. Time of incubation, 60 min.

Form of isotope	*Additions to incubation medium*	*% total radioactivity in form of*		
		Free base	*Nucleoside*	*Nucleotides*
[8-^{14}C]adenine	Nil	26	0	71
	Glucose (5.0 m*M*)	4	0	96
	Adenosine (0.5 m*M*)	2	0	96
	Uridine (0.5 m*M*)	8	0	91
[2-^{14}C]uracil	Nil	86	0	14
	Glucose (2.0 m*M*)	20	0	80
	Adenosine (0.5 m*M*)	13	1	86
	Uridine (0.5 m*M*)	39	2	59

or a nucleoside is added to the incubation medium, the proportion in the nucleotide form is substantially increased.

Rabbit bone marrow

The behaviour of rabbit bone marrow differs radically from that of the Ehrlich ascites carcinoma. Glucose and pyrimidine nucleosides have little or no effect on incorporation of [^{14}C]formate into nucleic acid adenine *in vitro* (Table I). A mixture of glucose, glutamine, aspartic acid and glycine produces a slight increase in incorporation which is more easily demonstrated if the cells are carefully washed with buffer before the start of the experiment (Table I). But even under these conditions the specific activities of DNA and RNA adenine remain very low relative to those of DNA thymine. Boiled rat liver extract, which, like glucose, stimulates incorporation of [^{14}C]formate into the purine nucleotides of the Ehrlich ascites carcinoma *in vitro*[3] does not produce an analogous effect on bone marrow. Blood serum, which also has a specific stimulating effect on purine nucleotide biosynthesis in the Ehrlich ascites tumour *in vitro*[3], stimulates incorporation of [^{14}C]formate equally into nucleic acid purines and DNA thymine *in vitro* (Table I). Finally, the ability of the bone marrow cells to incorporate [^{14}C]formate into their nucleic acid bases *in vitro* is very little impaired if the cells are incubated in buffer for up to 60 min before adding the isotope (Table I). This is in marked contrast to the Ehrlich ascites carcinoma, which, when similarly treated, shows a marked reduction in its capacity to incorporate [^{14}C]formate (Table I).

Other tissues

Incorporation of [^{14}C]formate has also been investigated in two other neoplastic tissues, the L5178Y ascites lymphoma and a transplantable mouse mammary adenocarcinoma (Table VII). In contrast to the results obtained with the Ehrlich ascites carcinoma, the activity of the nucleic acid adenine in the ascites lymphoma is quite comparable with that of the DNA thymine (Table VII). The effect of glucose on the ascites lymphoma is to increase incorporation into nucleic acid adenine and DNA thymine to approximately the same extent (Table VII). There is no suggestion of a specific effect on purine nucleotide biosynthesis. Addition of uridine does not increase incorporation into nucleic acid adenine (Table VII). These differences between the two ascites tumours do not appear to be attributable to differences in the amount of energy available to them. The relative proportions of ATP, ADP and AMP are roughly the same in both, and are similarly affected by incubation in presence and absence of glucose (Table II). Examination of the two tumours, however, shows that *in vivo* the lymphoma has a glucose content which varies from animal to animal but which is generally in the range of 10 to 40 mg/100 ml tumour. The corresponding figure for the Ehrlich ascites carcinoma is always less than 3 mg/100 ml. This difference *in vivo* is reflected in a difference in the rate at which the cells of the two tumours take up added glucose *in vitro*. Fig. 1 shows that the rate of uptake is very much more rapid in the carcinoma than in the lymphoma. The differences in the glucose requirement of the two tumours may in turn reflect differences in their mode of growth. Although the density of the cell population is greater in the lymphoma—approximately $170 \cdot 10^6$ cells per ml compared to approximately $80 \cdot 10^6$ cells per ml in the carcinoma—the carcinoma gives a much higher packed cell volume when centrifuged in a haematocrit

TABLE VII

INCORPORATION OF [^{14}C]FORMATE INTO THE ACID-SOLUBLE AND NUCLEIC ACID BASES OF AVIAN AND MAMMALIAN TISSUES *in vitro*

Initial isotope concentration 5 μC/ml.

Tissue	*Additions to incubation medium*	*Specific activity (counts/min/μmole)*			
		Acid-soluble adenine	*RNA adenine*	*DNA adenine*	*DNA Thymine*
L5178Y ascites lymphoma	Nil	—	7,790	2,900	10,840
	Glucose (5.0 m*M*)	—	22,100	8,540	31,780
	Uridine (0.5 m*M*)	—	8,650	1,488	10,400
Solid mammary adenocarcinoma	Nil	—	61,250	6,600	11,280
	Glucose (5.0 m*M*)	—	194,100	28,390	13,070
Adult rat brain	Nil	7,000	440	—	—
	Glucose (5.0 m*M*)	22,200	3,480	—	—
Chick embryo brain	Nil	89,000	7,700	1,630	15,500
	Glucose (5.0 m*M*), glutamine (0.5 m*M*)	88,000	9,400	1,650	28,300
Chick embryo skeletal muscle	Nil	14,600	1,300	494	30,300
	Glucose (5.0 m*M*), glutamine (0.5 m*M*)	128,000	10,300	2,220	45,800
Solid Ehrlich carcinoma	Nil	—	15,150	700	3,970
	Glucose (5.0 m*M*)	—	60,900	3,730	18,730
Solid L5178Y lymphoma	Nil	—	1,360	420	2,360
	Glucose (5.0 m*M*)	—	6,940	1,860	14,400

tube, 26 to 32% of the tumour cell suspension compared to 11 to 22% for the lymphoma. On the average, therefore, the mass of cells per ml of tumour cell suspension is about twice as high in the carcinoma as it is in the lymphoma.

To determine whether the *in vitro* responses to glucose of the two ascites tumours are due to inherited cell characteristics or merely to their relationship with their environment *in vivo*, both tumours were grown subcutaneously in the solid form. When slices of such tumours are incubated *in vitro* with [^{14}C]formate both give similar incorporation patterns in which the activity of the DNA thymine is approximately five times that of the DNA adenine (Table VII). In both cases addition of glucose to

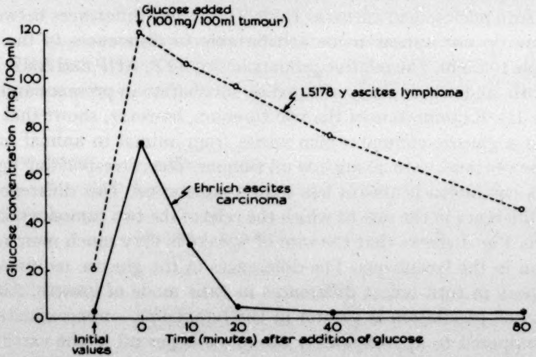

Fig. 1. Uptake of glucose by Ehrlich ascites carcinoma and L5178 Y ascites lymphoma *in vitro*.

the incubation medium increases incorporation into DNA thymine and DNA and RNA adenine to about the same extent (Table VIII).

The solid adenocarcinoma gives rather different results. *In vitro*, like the ascites lymphoma, it incorporates [^{14}C]formate extensively into nucleic acid adenine as well as into DNA thymine (Table VII). Glucose, however, produces a threefold increase in incorporation into nucleic acid adenine, without greatly affecting incorporation into DNA thymine. To determine whether other rapidly-growing normal tissues might behave in the same manner, the effects of glucose (supplemented with glutamine) on chick embryo brain and skeletal muscle was investigated (Table VII). In both cases the controls show much greater incorporation into DNA thymine than into nucleic acid adenine. Addition of glucose and glutamine increases the specific activity of DNA thymine by about 50 % in both. In skeletal muscle it also increases incorporation into acid-soluble and nucleic acid adenine 5- to 8-fold, but even under these circumstances the specific activity of the DNA adenine is still only about 5 % that of the DNA thymine. In embryo brain, on the other hand, glucose and glutamine appear to have no effect whatever on incorporation of [^{14}C]formate into acid-soluble and nucleic acid adenine. This is in sharp contrast to the situation in adult rat brain, where addition of glucose and glutamine more than trebles incorporation of [^{14}C]-formate into acid-soluble and RNA adenine *in vitro* (Table VIII).

TABLE VIII

DISTRIBUTION OF RADIOACTIVITY BETWEEN C-2 AND C-8 OF ACID-SOLUBLE ADENINE IN EHRLICH ASCITES CARCINOMA AND RABBIT BONE MARROW CELLS FOLLOWING INCUBATION FOR 4 H *in vitro* IN PRESENCE OF [^{14}C]FORMATE

	% *radioactivity*	
	C-2	*C-8*
Ehrlich ascites carcinoma	91	9
Rabbit bone marrow	61	39

DISCUSSION

The results described above go some way toward answering the three questions raised in the Introduction.

The influence of glucose on the Ehrlich ascites carcinoma

Two alternative hypotheses have been put forward to explain the fact that addition of glucose greatly increases purine biosynthesis in the Ehrlich ascites carcinoma *in vitro*.

1. HARRINGTON[6] has suggested that the glucose may act as a source of the ribose necessary for nucleotide biosynthesis. The principal evidence for this view has hitherto been the fact that low concentrations of cytidine and uridine (0.5 m*M*), which could liberate ribose-1-phosphate by the action of nucleoside phosphorylase, produce the same effect as much higher concentrations of glucose.

2. The validity of HARRINGTON's "ribose theory" has been challenged by HENDERSON AND LEPAGE[15], who have shown that the effects of glucose are abolished

by iodoacetate (alone or with dinitrophenol). From this they conclude that the "principal role of glucose is as an energy supply". The abolition of the glucose effect by iodoacetate is not reversed by methylene blue, which might be expected to increase oxidation of glucose and consequent production of pentose phosphate. This observation leads HENDERSON AND LEPAGE[15] to believe that the effect of glucose as a source of ribose is "a secondary consideration". The results of the present series of experiments support the first of these hypotheses rather than the second. It is quite clear from Tables II and III that when the Ehrlich ascites carcinoma is incubated aerobically *in vitro* the cells have a high content of ATP. Their failure to synthesize purines under these conditions cannot therefore be due to lack of the necessary energy. Moreover, under aerobic conditions glucose and uridine do not raise the ATP level, but tend rather to depress it. The increase in purine nucleotide biosynthesis which they produce cannot therefore be due to their acting as sources of energy. There remains the possibility that they are acting as sources of ribose.

Other evidence supports this opinion. The present experiments (Table III) have shown that when the tumour cells are incubated *in vitro* without glucose, their ATP content depends on the O_2 supply available. Under aerobic conditions it remains high, but under N_2 it falls off rapidly. In a previous paper[4] it was shown that *in vitro* increasing the amount of O_2 available to the cells also increased nucleic acid biosynthesis as indicated by incorporation of a wide range of precursors. This increase is presumably due to the effect of O_2 in maintaining the ATP content of the cells at a high level. Addition of glucose, on the other hand, which does not increase the ATP level of cells under aerobic conditions (Tables II and III), has little effect on nucleic acid biosynthesis (as indicated by incorporation of $^{32}PO4$ (see ref. 4)). It does, however, produce a great increase in purine nucleotide biosynthesis as indicated by [^{14}C]formate incorporation (Table I). It thus appears that oxygen supply determines the energy available to the cells and therefore the rate at which they synthesize nucleic acids; but that, even under aerobic conditions when the content of ATP in the cells is high, an external supply of glucose is needed for purine nucleotide biosynthesis. Under these circumstances the glucose is presumably required as a source, not of energy, but of ribose phosphate.

This conclusion is not inconsistent with the observation of HENDERSON AND LEPAGE[15] that iodoacetate abolishes the stimulatory effect of glucose on incorporation of [2-^{14}C]glycine into the acid-soluble adenine of the cells. Iodoacetate is not a highly specific enzyme inhibitor and even in absence of added glucose it depresses the ATP level of the cells during aerobic incubation (Table III). It has been shown above that under such conditions the cells contain no free glucose, but appear to maintain their ATP content by oxidative phosphorylation, probably at the expense of lactate and pyruvate. Iodoacetate presumably must inhibit some stage of this process. Its action therefore, in reversing the stimulating effects of glucose, does not prove that these effects are a result of glycolysis. It probably only reflects the fact that purine nucleotide biosynthesis requires ATP as well as ribose phosphate, without indicating the sources from which the ATP is derived. If this is so, it is not surprising that, as HENDERSON AND LEPAGE[15] have shown, the use of methylene blue (to increase glucose oxidation and hence the supply of ribose) fails to reverse the inhibitory effects of iodoacetate.

The theory that *in vitro* the external glucose supply determines the amount of ribose available for nucleotide biosynthesis in the Ehrlich ascites carcinoma is supported by the results in Tables V and VI. These show that the degree to which uracil, adenine and orotic acid are taken up by the cells *in vitro* under aerobic conditions is increased in presence of glucose or nucleosides, and that the increase is accompanied by increased conversion of the free bases to the corresponding nucleotides. Since under these conditions the cells have a high ATP content whether glucose and nucleosides are present or not (Tables II and III) the effects produced by these compounds are presumably due to their acting as sources, not of energy, but of the ribose phosphate necessary for nucleotide synthesis. Free ribose is ineffective, just as it is ineffective in stimulating purine biosynthesis *in vitro*[6]. Presumably the cells lack the ribokinase necessary to phosphorylate it[6]. In contrast, the uptake of glycine and phenylalanine under aerobic conditions is unaffected by addition of glucose (Table V). Under aerobic conditions, when the energy derived from oxidative phosphorylation is not available, uptake of phenylalanine is depressed. It can, however, be restored by addition of glucose, presumably because the latter can provide the necessary energy as a result of glycolysis (Table V). Uptake of phenylalanine, therefore, seems to depend on the energy available; uptake of adenine, uracil and orotic acid and their conversion to nucleotides depend also on the available supply of ribose phosphate.

Purine biosynthesis in rabbit bone marrow

At least two possible theories can be put forward to account for the very slow rate of purine nucleotide biosynthesis in rabbit bone marrow *in vitro*.

1. It is possible that *in vivo* the bone marrow might be supplied by the bloodstream with some precursor necessary for the synthesis (just as the Ehrlich ascites carcinoma is supplied with glucose); and that *in vitro* the absence of this precursor might diminish the rate of synthesis.

2. Alternatively, it is possible that the bone marrow cells may have only a very limited capacity for purine nucleotide biosynthesis and that *in vivo* they may obtain most of the purine they require from the bloodstream[16].

The present experiments give no support to the first of these hypotheses. Of the numerous precursors required for purine nucleotide biosynthesis[17], only glucose, glutamine, aspartic acid and glycine are likely to be present in the blood plasma, and it has been shown above that a mixture of all four has only a slight effect on incorporation of [^{14}C]formate into the purine nucleotides *in vitro* (Table I). Moreover, if purine biosynthesis in bone marrow *in vivo* depends on a supply of some precursor from the blood, addition of blood serum should increase purine biosynthesis in bone marrow cells *in vitro*. Table I, however, shows that blood serum has no specific effect on incorporation of [^{14}C]formate into nucleic acid purines, which is increased only to the same extent as incorporation into DNA thymine. Blood serum therefore appears, as THOMAS has already shown[18], to contain factors which stimulate biosynthesis of nucleic acids but not of purine nucleotides. This is in contrast to the Ehrlich ascites carcinoma, in which purine nucleotide biosynthesis is specifically increased by small amounts of blood serum[3], presumably because of its glucose content. Moreover, boiled liver extract, which also stimulates purine biosynthesis in the ascites tumour[3], and which might be expected to contain a wide variety of purine precursors, has no effect on bone marrow.

The second theory is more consistent with the marked differences in purine biosynthesis *in vitro* between bone marrow and the Ehrlich ascites carcinoma found in the present experiments. Thus the very limited incorporation of [^{14}C]formate into acid-soluble purines in the Ehrlich ascites tumour *in vitro* in absence of glucose is confined chiefly to the first 30 min of incubation (Fig. 2). If addition of the isotope is delayed beyond this period, incorporation is greatly diminished (Table I). Even if there is no delay in addition of the isotope, incorporation is confined chiefly to the C-2 position (Table VIII). Since relatively little activity is found in C-8, it is probable that incorporation may be due chiefly to conversion of existing 5-amino-4-iminazolecarboxamide ribonucleotide to purine nucleotides (or to labeling of existing purine nucleotides by transformylation[15]) rather than to *de novo* purine nucleotide biosynthesis from glycine and phosphoribosyl pyrophosphate. This conclusion is supported by the relatively small decrease in incorporation brought about by azaserine, which is known to inhibit one of the early stages of purine nucleotide biosynthesis (Table IX). These observations suggest that in this tissue [^{14}C]formate incorporation into purines *in vitro* represents the last stage of a process of purine biosynthesis which is being brought to a standstill by lack of one of the precursors (glucose) necessary for its continuance.

In bone marrow, on the other hand, incorporation of [^{14}C]formate into acid-soluble purines, although slow, continues steadily for up to 4 h (Fig. 2), and the isotope incorporated is fairly equally distributed between the C-2 and C-8 positions of the purine nucleus (Table IX). Incorporation seems therefore to represent biosynthesis of purines *de novo* from glycine and phospho-ribosylamine, rather than from such intermediates as 5-amino-4-iminazolecarboxamide ribonucleotide. In agreement with this hypothesis, azaserine has a greater inhibitory effect on incorporation in bone marrow than in the Ehrlich ascites tumour (Table IX). Again in contrast to the ascites tumour, incorporation is not greatly diminished if addition of the isotope is delayed until, say,

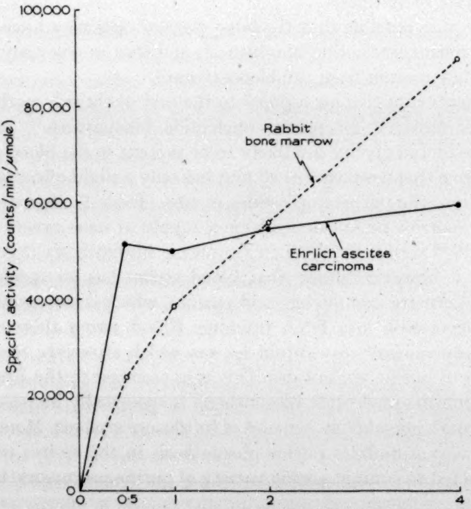

Fig. 2. Incorporation of [^{14}C]formate into acid-soluble adenine of rabbit bone marrow and Ehrlich ascites carcinoma cells *in vitro*.

TABLE IX

EFFECT OF AZASERINE ON INCORPORATION OF $[^{14}C]$FORMATE INTO THE NUCLEIC ACID BASES OF MAMMALIAN TISSUES *in vitro*

Results are expressed as percentages of control values.

	RNA adenine	*DNA adenine*	*DNA thymine*
Ehrlich ascites carcinoma	50	60	86
Rabbit bone marrow	20	19	89
Solid mammary adenocarcinoma	2	4	85
L5178Y ascites lymphoma	11	5	100

1 h after the beginning of the incubation (Table I). It therefore appears that removal of bone marrow from the animal and its subsequent incubation *in vitro* may not produce any very drastic change in its capacity for purine nucleotide biosynthesis. If this is so, bone marrow *in vivo* must synthesize purines relatively slowly and must depend largely on a supply of preformed purines from the blood.

Purine biosynthesis in other tissues

It has previously been shown that a low rate of purine biosynthesis *in vitro* (in absence of glucose) is found, not only in rabbit bone marrow and the Ehrlich ascites carcinoma, but also in a wide range of rapidly growing tissues[3]. The results in Table VII, however, show that this is not an invariable characteristic of such tissues. Thus the L5178Y ascites lymphoma, which *in vivo* grows at approximately the same rate as the Ehrlich ascites carcinoma, appears *in vitro* to synthesize purines much more rapidly (Tables I and VII). On the evidence presented above, this difference can be explained entirely in terms of glucose requirement. Both tumours can synthesize purines rapidly *in vitro* if provided with glucose. *In vivo*, however, the ascites lymphoma makes smaller demands on the supply of glucose available in the peritoneal cavity. Consequently it has a reserve of glucose, whereas the ascites carcinoma has none. Hence removal from the body of the host, with consequent cessation of glucose supply, has an inhibitory effect on purine biosynthesis in the carcinoma, but not in the lymphoma. When the two ascites tumours are grown in solid form, their fundamental similarity is strikingly illustrated. Slices of such tumours incubated *in vitro* in presence of $[^{14}C]$formate give essentially the same incorporation pattern (Table VII), in which the specific activity of the DNA adenine is about 20% that of the DNA thymine. The responses of the two solid tumours to added glucose are exactly similar. Incorporation into both DNA thymine and nucleic acid adenine is increased approximately 4- to 6-fold (Table VIII). There is no indication of a specific effect on purine biosynthesis.

The solid mammary adenocarcinoma fits into the same pattern as the two ascites tumours since, if supplied with additional glucose, it can incorporate $[^{14}C]$formate extensively into nucleic acid adenine *in vitro* (Table VII). It may indeed be regarded as intermediate between them, resembling the lymphoma in its ability to synthesize purines in absence of any addition, and resembling the carcinoma in responding to glucose by specifically increasing this incorporation. Such a relationship between glucose supply and purine biosynthesis is not limited to neoplastic tissues. Table VIII shows that purine biosynthesis *in vitro* in adult rat brain, a tissue with small reserves of glucose or glycogen, is substantially increased by addition of glucose and glutamine to the incubation medium. The behaviour *in vitro* of the chick embryo tissues, on the other hand, resembles that of bone marrow in that, even if they are supplemented with glucose and glutamine, their incorporation of [^{14}C]formate into DNA adenine is much lower than incorporation into DNA thymine (Table VIII). The presence of glucose and glutamine does not affect purine biosynthesis in chick embryo brain; in chick embryo muscle it produces some increase.

In conclusion, therefore, it appears that the tissues investigated so far fall into two main classes.

1. Those which, at least when supplied with glucose, are capable of incorporating [^{14}C]formate *in vitro* into DNA adenine and DNA thymine to approximately equal degrees (*i.e.* the Ehrlich and L5178Y tumours in both solid and ascites forms, the solid mammary adenocarcinoma, chick embryo liver and regenerating rat liver[3]).
2. Those which, even if supplied with purine precursors, do not incorporate [^{14}C]formate *in vitro* as extensively into DNA adenine as into DNA thymine (*i.e.* rabbit bone marrow and chick embryo brain and muscle).

Both classes include examples of tissues in which purine biosynthesis *in vitro* is increased in presence of glucose. This phenomenon therefore does not appear to be related to the ability of a tissue to synthesize for itself all the purine nucleotide which it requires. It seems rather to reflect the degree to which *in vivo* it depends on a continuous supply of glucose.

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STUDIES ON THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID BY EXTRACTS OF MAMMALIAN CELLS

IV. THE PHOSPHORYLATION OF THYMIDINE

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SUMMARY

Kinases responsible for the formation of thymine-deoxyribose-5′-monophosphate, diphosphate and triphosphate from thymine-deoxyribose have been purified from extracts of Ehrlich ascites carcinoma.

These enzymes are also found in rabbit tissues, highest levels of activity being observed in thymus and bone marrow.

In rat liver regenerating after partial hepatectomy thymine-deoxyribose, thymine-deoxyribose-5′-monophosphate and diphosphate kinases appear in that order, maximum activities being observed between 30 and 48 h after the operation.

In cultures of L. strain fibroblasts there is again a sequential appearance of thymine-deoxyribose, thymine-deoxyribose-5′-monophosphate and diphosphate kinases during the early part of the growth phase and the activities of all three enzymes decline before the completion of growth. Growth of L. strain fibroblasts in a medium containing thymine-deoxyribose lead to increases in kinase activities which were compatible with enzyme induction.

INTRODUCTION

In previous papers[1-3] we have described an enzyme system in cell free extracts of Ehrlich ascites carcinoma cells which promotes the incorporation of [^{3}H]TdR into carrier DNA and have shown that this process involves the phosphorylation of TdR to the corresponding triphosphate. The present paper describes the kinase systems responsible for such phosphorylation in extracts of ascites cells and of other mammalian tissues such as regenerating rat liver and strain L fibroblasts. Similar enzymes have been described in regenerating rat liver by POTTER *et al.*[4,5] by MANTSAVINOS AND CANELLAKIS[6] and by HIATT AND BOJARSKI[7].

Abbreviations: DNA, deoxyribonucleic acid; TdR, thymidine (thymine-deoxyribose); TMP, TDP and TTP, thymidine 5′-mono-, di-, and tri-phosphates respectively; ATP, adenosine triphosphate; DNase, deoxyribonuclease; Tris, 2-amino-2-hydroxymethyl propane-1, 3-diol. TdR kinase, TMP kinase and TDP kinase denote respectively the enzymes systems responsible for the formation of TMP, TDP and TTP.

MATERIALS AND METHODS

Enzyme assay

5.5 μmoles $MgCl_2$, 5.5 μmoles ATP, 110 μmoles Tris buffer pH 7.9 and 0.5 μC [^{3}H]TdR were incubated in 25-ml flasks for 90 min at 37° with an amount of enzyme corresponding in activity to 0.5 ml whole ascites extract. The final volume was 1.1 ml. The contents of the flasks were then transferred to chilled centrifuge tubes, 0.15 ml 4 N $HClO_4$ was added and the precipitated protein centrifuged down. All manipulations were carried out in the cold. The supernatant fluid was brought to pH 7.8 with KOH and the precipitated $KClO_4$ removed by centrifugation. 1 ml of the supernatant fluid was applied to an ECTEOLA cellulose column[8] (0.030 mequiv./ml) 8 cm × 1 cm. The column was eluted successively with 50-ml portions of water, 0.01 N HCl, 0.05 N HCl and 0.5 N HCl to remove TdR, TMP, TDP and TTP respectively. The columns were regenerated between runs by washing with 125 ml water and then resuspending the material in 25 ml water and allowing the column to drain.

0.2-ml portions of each 50-ml eluate were plated on stainless steel planchets and the time required for 500 counts determined in a Nuclear Chicago windowless gas flow counter. The total counts/min in each of the column eluates are expressed as a percentage of the total counts/min recovered from the column. The incorporation of [^{3}H]TdR into DNA was followed in some experiments by the method previously described[1].

Biological material

Cell free extracts were obtained from osmotically disrupted Ehrlich ascites tumour cells as previously described[1].

Rat and rabbit tissues were quickly removed from the exsanguinated animals, blotted with filter paper, finely minced with scissors in an ice-cooled beaker and disintegrated in a Potter type homogenizer, usually in 20 ml water for each liver or in 10 ml water for other organs. The homogenates were centrifuged at 105,000 × g for 1 h and the cell and particle-free extracts collected. The protein concentrations were adjusted to 3–6 mg/ml except in the case of liver extracts which usually had a protein concentration of 15–20 mg/ml.

In experiments with regenerating rat liver partial hepatectomy was kindly performed by Dr. R. Y. THOMSON by the method of HIGGINS AND ANDERSON[9].

Cell cultures

Adult mouse subcutaneous fibroblasts NCTC strain L clone 929 (see ref. 10) were grown in accordance with the methods described by PAUL[11].

The cells (1–20 million) were harvested by incubating for 5 min at 37° with 10 ml trypsin solution and where necessary by scraping the flask with a rubber-tipped glass rod. The cells were washed twice with 0.9 % NaCl solution and suspended in 0.25–1.0 ml water. A portion of the cell suspension was diluted with 0.9 % NaCl and counted in an automatic particle counter. The remainder was disrupted by exposure for 90 sec to the vibrations of a Mullard ultrasonic drill (20 kc./sec). A portion of the disruptate containing 0.25–1.0 mg protein was used for each assay. The same amount of protein was used in each flask.

Protein determinations in all cases were carried out by the method of LOWRY *et al.*[12].

[^{3}H]TMP was prepared by exposure of 250 mg calcium thymidylate to 7 C tritium gas in an 83 % mixture with hydrogen for 17 days. This was carried out at the Atomic Energy Research Establishment, Harwell, England. Subsequently the TMP was purified by lyophilizing several times from a large volume of water to remove labile tritium. It was then fractionated on a Dowex-1-Cl column and further purified by paper chromatography in ammonium isobutyrate[2], butanol–water[13] and ethanol–ammonium acetate[14] until all the radioactivity was found to be associated with the area corresponding to TMP. The final product was converted to the sodium salt by passage through a Dowex-50-Na column and had a specific activity of 10^7 counts/min/μmole as determined in a counter of approx. 30 % efficiency.

RESULTS

Enzyme purification

All manipulations were carried out in the cold.

Precipitation with acid: The supernatant fluid obtained by high speed centrifugation of the ascites tumour cell extract (containing 3–4 mg protein/ml) was brought to pH 4.5 with 1 *N* acetic acid. The precipitate was centrifuged down and the supernatant fluid brought to pH 7 with 1 *N* NaOH. This fluid (fraction AS 4.5) contained all the TMP kinase and TDP kinase and generally more than 66 % of the TdR kinase, and since the total protein concentration was 0.9–1.1 mg/ml, an approximately three-fold purification of TMP kinase and TDP kinase was achieved by this step (Table I).

Ammonium sulphate fractionation: To each 100 ml of AS 4.5, 21.5 g ammonium sulphate were added (final pH 6.4) to give approx. 35 % saturation. The solution was allowed to stand for 30–60 min, and the precipitate centrifuged down and redissolved in two-thirds of the original volume of water or of 0.01 *M* Tris buffer pH 7.8–7.9 giving fraction ASP 35 of protein concentration 0.15–0.30 mg/ml. It retained more than two-thirds of the TdR kinase activity of the AS 4.5.

Precipitates obtained by adding 19–21.5 g ammonium sulphate to the AS 4.5 showed variable retention of TMP kinase activity and could promote the formation of some TDP but not TTP. Similar results were obtained with extracts of rabbit bone marrow and thymus and in some instances with liver. Occasional preparations retained two-thirds or more of the original TMP kinase activity and showed a 15–20 fold

TABLE I

THYMIDINE KINASE PURIFICATION FROM ASCITES CELL EXTRACT

	*Total units**	*Specific activity***
Extract	387	70
AS 4.5	410	171
ASP 35	160	960
ASP 35 ECTEOLA fraction	$< 20–25$	> 2500

* Per cent TdR phosphorylated for appropriate dilution.
** Per cent TdR phosphorylated/mg protein/ml.

purification. This enzyme was very unstable at this stage and storage overnight led to marked loss of activity. TDP kinase was not detectable either in the precipitate or in the dialysed supernatant fluid. In certain experiments the supernatant fluid obtained on adjusting the pH of the whole ascites extract stepwise to 4.25–4.0 showed ability to form TTP when added to preparations of TdR kinase plus TMP kinase which in themselves had no TDP kinase activity.

Column fractionation: 5 ml of fraction ASP 35 dissolved in water were applied to a column of ECTEOLA cellulose 3 cm × 1 cm. When the fluid had reached the surface of the column, 5 ml 0.01 *M* Tris buffer pH 7.8 were passed through the column followed by a 5-ml portion of 0.1 *M* Tris buffer pH 7.7. The total activity recovered was about 10–40 % of that applied in less than 20 μg protein. The final purification of TdR kinase was approx. 20–40 fold. Similar results were obtained with DEAE cellulose columns eluted with 0.1 *M* and 0.2 *M* Tris buffer.

Attempts at acetone fractionation or treatment with calcium phosphate gel resulted in marked loss of activity.

Properties of the kinases

Confirmation that the nature of the product of the action of TdR kinase was in fact TMP-5′ was obtained by paper chromatography and by treatment with snake venom 5′-phosphomonoesterase.

All activity disappeared on heating to 65° for 5 min or on altering the pH to above 8.25 or below 4.25. TDP kinase was slightly more acid resistant than TdR kinase or TMP kinase. Under storage at $-10°$ activity declined over a period of weeks.

TdR kinase was stable to dialysis but TMP kinase and TDP kinase lost activity on contact with unwashed dialysis tubing. Consequently dialysis tubing was always washed several times with water, filled with distilled water and soaked for several hours in distilled water before use. With these precautions TMP kinase was entirely stable, and TDP kinase partially stable, in dialysis for several hours against 0.1 *M* Tris buffer pH 7.8–7.9 containing 1 μmole 2-mercaptoethanol/ml and 1 mμmole TMP/ml.

TdR kinase showed optimum activity at pH 7.9 but TMP kinase activity and especially TDP kinase activity tended to decline above pH 7.7.

Mg ions were necessary for activity and kinase activities were maximal in the presence of 5 μmoles/ml of both Mg^{++} and ATP. When the concentration of Mg^{++} and ATP was reduced from 5 μmoles/ml to 1 μmole/ml, the formation of TDP and TTP was diminished without affecting TMP formation. In the presence of optimal concentrations of Mg^{++} and ATP, increasing concentrations of one of several ions (including Tris) tended to decrease yields of TDP and TTP (Table II). Phosphate ions tended to decrease the total yield of thymidine nucleotides.

Studies on the course of formation of phosphorylated derivatives indicated that the formation of TMP and TDP preceded that of TTP. The absolute amounts of substrate phosphorylated by whole ascites extract increased with increasing thymidine concentration between 1 and 50 mμmole/ml. Formation of [^{3}H]TTP from [^{3}H]TMP over this concentration was a nearly constant proportion of the substrate. While the absolute amount of phosphorylation increased slightly with increasing concentrations of TdR, the proportion of TdR phosphorylated decreased markedly. Preincubation of the enzyme at 37° for 30 min without substrate caused marked loss of activity.

The curve in Fig. 1 shows an approximately linear relationship between enzyme concentration and the extent of phosphorylation over the range 25 to 65 % conversion to phosphorylated derivatives.

Distribution

Kinase activities were found in varying degrees in several tissues (Table III). TMP kinase and TDP kinase in particular tended to be associated, so that no tissue showed a preponderant production of TDP.

Kinases in extracts of regenerating rat liver

As has been mentioned by other workers[4–7] a marked rise in kinase activity in liver tissue occurs during regeneration after partial hepatectomy. The livers of rats

TABLE II

EFFECTS OF VARIOUS IONS ON KINASES

Test substance	*µmoles/ml*	*Per cent formed*		
		TMP	*TDP*	*TTP*
$MgCl_2$ control	5	10	10	69
$MnCl_2$	10	9	17	61
KCl	10	6	15	65
KCl	100	22	17	44
$CaCl_2$	10	16	19	39
$PO_4^{\equiv}$ (Na buffer pH 7.9)	10	4	20	52
$PO_4^{\equiv}$ (Na buffer pH 7.9)	50	3	29	25
NaCl	10	6	25	44
NaCl	100	23	26	31
$(NH_4)_2SO_4$	10	17	15	40
$(NH_4)_2SO_4$	100	44	10	32
NH_4Cl	10	12	29	16
NH_4Cl	50	48	23	4
$FeCl_3$	2	6	51	19
K_2SO_4	10	5	11	64
K_2SO_4	50	6	48	22

TABLE III

DISTRIBUTION OF KINASES IN RABBIT TISSUES

	Per cent formed		
	TMP	*TDP*	*TTP*
Plasma	4	1	0
Erythrocytes	7	1	1
Leucocytes (buffy coat)	9	12	8
Muscle	8	1	1
Brain	17	4	2
Intestine	27	15	6
Liver	17	7	1
Kidney	18	5	3
Spleen	27	12	14
Thymus	8	18	55
Bone marrow	10	16	49

killed 6 and 18 h post-operatively show no higher enzyme levels than did those of control animals but a distinct elevation in TdR kinase and TMP kinase activity was found at 24 h (Fig. 2). A few animals showed elevation of TdR kinase activity only. The peak in TDP kinase activity occurred between 30 and 48 h post-operatively. This correlated well with the results of experiments on the rate of incorporation of [^{3}H]TdR into DNA.

The precipitate obtained from extracts of normal liver by saturation with ammonium sulphate to 25 % represented 8 % of the protein of the extract and contained most of the TdR kinase. Since such a precipitate from extracts of 48 h regenerating liver was at least 10 times more active, there is good evidence for an increase in the amount of enzyme.

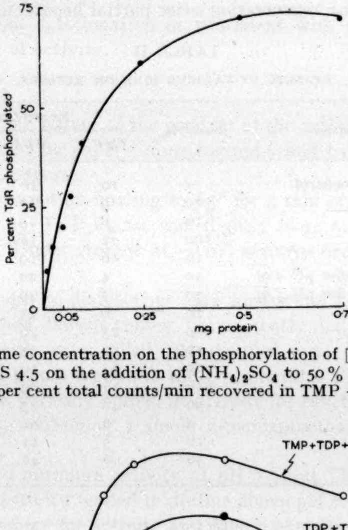

Fig. 1. The effect of enzyme concentration on the phosphorylation of [^{3}H]TdR by the precipitate obtained from fraction AS 4.5 on the addition of $(NH_4)_2SO_4$ to 50 % saturation. The results are expressed as per cent total counts/min recovered in TMP + TDP + TTP.

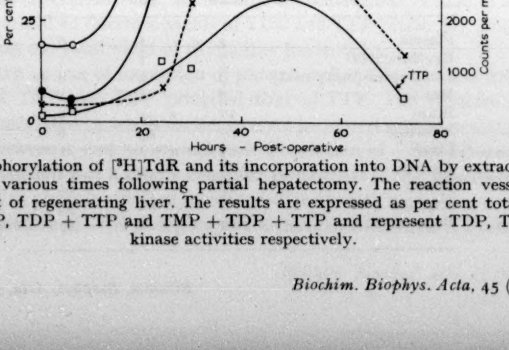

Fig. 2. The phosphorylation of [^{3}H]TdR and its incorporation into DNA by extracts of rat liver regenerating for various times following partial hepatectomy. The reaction vessels contained 0.6 ml of extract of regenerating liver. The results are expressed as per cent total counts/min recovered in TTP, TDP + TTP and TMP + TDP + TTP and represent TDP, TMP and TdR kinase activities respectively.

An attempt was made to induce TdR kinase in rat liver according to the method of HIATT AND BOJARSKI[7] by hourly intraperitoneal injections of 100 mg TdR. Animals were killed 2, 4 and 6 h after the first injection and liver extracts assayed both before and after dialysis. No detectable change in liver enzyme levels was found.

Kinases in cell cultures

L. strain mouse fibroblasts in the resting phase after exhaustion of the medium showed either no kinase activity whatever or only TdR kinase activity. On inoculation into a fresh medium, a period of rapid growth occurred after a lag phase (Fig. 3). TMP kinase activity rose slightly before the onset of rapid growth and was followed by TDP kinase. TDP kinase activity also declined earlier, its peak corresponding to the early portion of the rapid growth phase. Similarly in experiments in which growth began and ended sooner after inoculation, there was an earlier rise and decline in kinase activities.

When alternate flasks of L. cells were treated with TdR on day 0 at a final concentration of 10^{-4} *M* and replenished with the same amount on day 4, no appreciable differences in enzyme activity levels between treated and control flasks were observed for the first 3 days (Fig. 4). Although the growth rates were identical in control and treated flasks throughout, TdR kinase activity did not decline so rapidly in the treated cells as in the controls while TMP and TDP kinases showed a second and prolonged peak of activity. All levels fell at the end of the experiment.

In other experiments inocula of $3 \cdot 10^6$ rather than $5 \cdot 10^6$ cells were used. TdR was added either at time 0 or after 3 days of growth to a final concentration of 10^{-4} *M*. After 7 days the medium was replaced with fresh medium containing TdR where appropriate, so as to permit continuous growth of the cultures for 9 days.

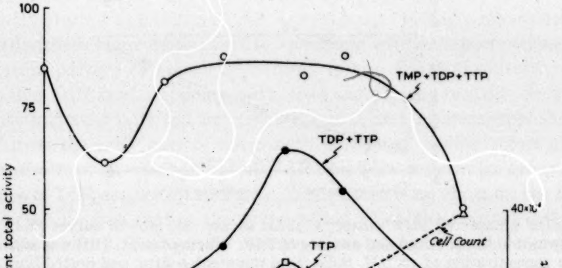

Fig. 3. The phosphorylation of [^{3}H]TdR by disrupted preparations of L strain mouse fibroblasts at various times during growth. Each reaction vessel contained 1.0 mg total protein. Results are expressed as per cent total counts/min recovered in TTP, TDP + TTP and TMP + TDP + TTP representing TDP, TMP and TdR kinases respectively.

In this group of experiments the TdR concentration proved to be mildly toxic so that the treated cells grew more slowly than the controls. The activities of TMP and TDP kinases rose earlier in the cells treated with TdR at time 0 than in the more rapidly growing controls, remained elevated until shortly before the growth rate declined and then fell sharply.

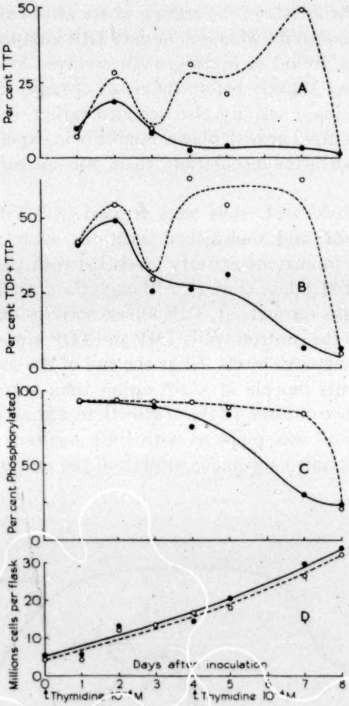

Fig. 4. A, TDP kinase; B, TMP kinase; C, TdR kinase; D, growth curves of L. strain mouse fibroblasts grown in the presence and absence of TdR. Where present, TdR was added at the times shown, to a concentration of 10^{-4} *M*. Solid lines represent control and dotted lines test cultures. The total protein per reaction vessel was between 1.0 and 1.5 mg.

In the group of cells where TdR was added after 3 days there was a reduction in rate of growth. Four days after addition of TdR there was a fall in TMP and TDP kinase activities and in the rate of growth. This was followed, however, by a second rise in cell count and in TMP and TDP kinase activities.

In both test and control cultures the TDP kinase activity tended to fluctuate more and to parallel the growth rate more closely than did the TMP kinase. TdR kinase remained elevated through most of the growth phase in all cultures.

DISCUSSION

The results of fractionation experiments on extracts of Ehrlich ascites cells have shown that three enzymes are involved in the formation of TTP from TdR. TdR kinase has been purified 20–40 times by acid precipitation followed by ammonium sulphate fractionation and chromatography on columns of ECTEOLA or DEAE cellulose. TMP kinase proved to be extremely labile but 15–20 fold purification was obtained in some samples by acid precipitation followed by ammonium sulphate fractionation. TDP kinase also proved highly labile although 2–3 fold purifications were regularly obtained by removal of inactive protein by acid precipitation.

The TMP kinase appears to be quite distinct from the relatively non-specific nucleoside monophosphate kinase described by STROMINGER *et al.*[15] since it is much more labile, has a much lower reaction velocity and behaves differently on ammonium sulphate fractionation. The bacterial TMP kinase of HURWITZ[16] also differs from the ascites enzyme in its precipitation with ammonium sulphate and in its stability.

It has previously been shown that TTP may be formed from TdR by preparations from regenerating rat liver[4,5,7,17–20] and that regenerating liver differs from normal liver in possessing the necessary kinases[4,7,20]. The results in Fig. 3 show that the appearance of the kinases after partial hepatectomy tends to be sequential, TdR and TMP kinases appearing before TDP kinase. By 24 h the TdR and TMP kinases are increasing sharply reaching a maximum of activity around 30 h. TDP kinase activity develops more slowly and reaches a peak at about 48 h. Measurements of the incorporation of [^{3}H]TdR into DNA show little difference between normal and regenerating liver until about 30 h after partial hepatectomy, and maximum uptake is observed at about 48 h. Subsequently all kinases decline in activity. In the experiments of BOLLUM AND POTTER[4] the greatest uptake of TdR into DNA was observed between 24 and 60 h following partial hepatectomy and this is substantially confirmed by our findings. TMP kinase activity has been studied by HIATT AND BOJARSKI[7] who found that the activity started to rise at 20 h post-operatively, reached a maximum at about 42 h and then declined. These findings are in agreement with our observations in Fig. 3.

A sequential pattern of kinase appearance is also found in cultures of L. cells. Fig. 3 shows that TdR kinase appears early rises sharply and remains elevated. TMP kinase activity increases later but before TDP kinase and both enzymes show a peak of activity during the early part of the growth phase and decline before growth has stopped. The results of an effort to induce TdR and TMP kinases by growing L. cells in the presence of TdR are shown in Fig. 4. While there is no effect on the net rate of growth or on the kinases during the early period when the control cultures show maximal kinase activity, TdR kinase remains elevated for a much longer period in the test cultures and TMP and TDP kinases rise to a second peak during the period when these activities in the control cultures decline. These differences between control and test culture were observed only during growth.

These results, together with those from other experiments reported in the results section, suggest that such elevations in kinase activities can only be produced during the growth phase and could be due to true enzyme induction. An alternative explanation that the results might be due to *in vivo* protection of the enzymes by their respective substrates seems less likely in view of the observation that enzyme levels fell sharply when cell growth ceased but could rise equally sharply on replenishment of the medium irrespective of the length of exposure to TdR.

Attempts to induce TdR kinase in rat liver by the methods which HIATT AND BOJARSKI[7] employed to induce thymidylate kinase proved unsuccessful. These, however, were short term experiments.

The results presented here are consistent with the possibility of sequential induction of enzymes concerned with the synthesis of TTP and support the view that enzymes converting TdR to TTP are specifically concerned with processes relating to cell division.

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STUDIES ON THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID BY EXTRACTS OF MAMMALIAN CELLS

V. FACTORS INTERFERING WITH BIOSYNTHESIS

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SUMMARY

Cell free extracts of certain tissues such as Ehrlich ascites carcinoma, regenerating rat liver and rabbit bone marrow contain kinases which promote the conversion of thymidine to the mono- di- and triphosphates and an enzyme which catalyses the polymerization of the four deoxyribucleoside triphosphates into DNA.

Such enzymes are much less abundant in extracts of non-proliferating tissues such as liver and kidney.

The addition of extracts of liver or kidney greatly reduces the ability of extracts of ascites cells, bone marrow or regenerating rat liver to phosphorylate thymidine and to form DNA.

Extracts of normal liver and of ascites cells contain kinases which bring about the phosphorylation of the monophosphates of deoxyadenosine, deoxyguanosine, and deoxycytidine. The addition of liver extracts to ascites extracts causes no reduction in kinase activity in such systems.

INTRODUCTION

In previous communications [1–4] we have reported the presence in cell free extracts of Ehrlich ascites carcinoma cells and other mammalian tissues, of kinases which bring about the phosphorylation of TdR to TTP and of an enzyme which promotes the polymerization of the four deoxyribonucleoside triphosphates to form DNA in a manner similar to that described by KORNBERG *et al.*[5] for *E. coli*. Such enzymes are also present in extracts of regenerating rat liver[6–9].

The present paper describes the effects of extracts of normal liver tissue on the kinases and polymerases from other tissues. A preliminary report has already appeared[10].

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; dAMP, dGMP, dCMP and TMP, 5′-monophosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine respectively; dATP, dADP, dGDP, dGTP, dCTP, dCDP, TTP and TDP, 5′-tri- and diphosphates respectively of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine; TdR, thymidine; ATP, adenosine-5′-triphosphate; DPN, diphosphopyridine nucleotide; DNase, deoxyribonuclease; RNase, ribonuclease; TCA, trichloroacetic acid; Tris, 2-amino-2-hydroxymethyl propane-1, 3-diol.

METHODS

Biological

The Ehrlich ascites carinoma, kindly supplied by Dr. G. POPJÁK of Hammersmith Hospital, London, was maintained by serial transplantation in mice of the departmental colony. Rabbits weighing approximately 2000 g and rats of about 200 g were obtained from the departmental colonies. Partial hepatectomies were kindly performed by Dr. R. Y. THOMSON by the procedure of HIGGINS AND ANDERSON[11].

Preparation of tissue extracts

All manipulations were carried out in the cold. Extracts of Ehrlich ascites tumour cells were prepared from osmotically disrupted cells by centrifuging at 105,000 × *g* as previously described[1]. Extracts of rabbit tissues were prepared by homogenising in 10 volumes distilled water or 0.1 *M* Tris buffer pH 7.9 for 2 min in a Potter type of homogeniser. Rat livers were homogenised in 20 ml distilled water for 2 min in a Potter type homogeniser. Both types of homogenate were centrifuged at 105,000 × *g* for 1 h and the supernatant fluid decanted.

In some experiments liver extracts or fractions prepared from them were heated at 100° for 5 min and centrifuged for 10 min at 600 × *g* to remove precipitated protein.

Dialysis of tissue extracts or fractions was carried out for varying times against 0.1 *M* Tris buffer pH 7.9, 0.001 *M* with respect to 2-mercaptoethanol.

Preparation of deoxyribonucleoside 5′-triphosphates

Some samples of dATP and dGTP were prepared by a modification of the biological method of KEIR AND SMELLIE[2] as follows: A reaction mixture was set up containing the following components in a total volume of 120 ml: 300 μmoles dAMP (sodium salt), 900 μmoles ATP (sodium salt), 10 mmoles Tris buffer pH 7.9, 900 μmoles $MgCl_2$, 400 mg lyophilised Ehrlich ascites extract. The mixture was incubated at 37° for 7 h with shaking and was then heated to 100° for 3 min and rapidly cooled to 0°. The precipitate of protein was removed by filtration through Whatman No. 1 paper at 0°. Samples of the filtrate were taken for paper chromatography in the ammonium isobutyrate system[2] to obtain a measure of the extent of conversion to the triphosphate. The bulk of the solution was diluted to 2 l with distilled water and applied to a column of Dowex-1-Cl, 31 cm × 2 cm. After washing with water the column was eluted successively with 0.003 *N* HCl, 0.01 *N* HCl–0.02 *M* LiCl and 0.01 *N* HCl–0.2 *M* LiCl to bring off peaks corresponding to AMP + dAMP, ADP + dADP and ATP + dATP respectively. The tubes corresponding to ATP + dATP were pooled, neutralised with LiOH and concentrated to small volume in an Edwards Model 10P freeze drier. An equal volume of methanol and 20 volumes of acetone were added and the mixture cooled to —10° for 1 h to precipitate the lithium salts of ATP and dATP. These were collected by centrifugation and dried in air. The lithium salts were dissolved in distilled water and a sample taken for chromatography in the ammonium isobutyrate system to check that only ATP and dATP were present. The main bulk of the solution was made up to 55 ml and half of it taken for periodate oxidation to destroy ATP. 11.8 ml 0.1 *M* glycylglycine buffer pH 7.4 were added followed by 900 μmoles $NaIO_4$ and the mixture was incubated at 25° for 30 min with frequent shaking. Excess periodate was removed by adding 1.2 mmoles glucose followed by incubation for 30 min at 25°. The pH was adjusted to 10 by the addition of 10.4 ml *M* glycine buffer pH 10.2 and the solution incubated for 14 h at 37°. The alkaline incubation mixture was diluted to 350 ml with water and applied to a Dowex-1-Cl column, 13.5 cm × 2 cm. The column was washed with water (80 ml) and elution was carried out with 0.01 *N* HCl–0.02 *M* LiCl, 0.01 *N* HCl–0.08 *M* LiCl and 0.01 *N* HCl–0.20 *M* LiCl. This last solvent brought off the dATP and the tubes containing this were pooled, neutralised with LiOH, and the lithium salt precipitated with methanol and acetone after reduction of the volume. The lithium salt was dried in air, dissolved in water, a sample taken for chromatography and the remainder converted to the sodium salt by passage through a Dowex-50-Na column. The other half of the mixture of lithium salts of ATP and dATP was treated in the same manner and the products pooled. Paper chromatography revealed only one major spot corresponding to dATP and having a phosphorus:deoxyadenosine ratio[2] of 2.98:1. The total yield of dATP by this procedure was 200 µmoles.

Preparations of dGTP were made in the same manner except that it was not necessary to carry out the periodate oxidation to remove ATP since dGTP and ATP can be separated on Dowex-1-Cl columns. All the nucleotides except dGTP were eluted with 0.1 *N* HCl–0.1 *M* LiCl and the dGTP was eluted with 0.01 *N* HCl–0.2 *M* LiCl. The ratio of phosphorus to deoxyguanosine in the final product was 2.97:1 and on paper chromatography in ammonium isobutyrate it migrated as a single spot.

Other specimens of dATP and dGTP were prepared by a slight modification of the method of SMITH AND KHORANA[12] from dAMP and dGMP. dCTP and TTP were also prepared chemically from dCMP and TMP respectively by the SMITH AND KHORANA[12] procedure. TDP was obtained as a by-product of TTP synthesis.

TTP labelled in the proximal position with ^{32}P (TdR-^{32}P-P-P) was prepared from [^{32}P]TMP by the same method. For the preparation of [^{3}H]TTP, [^{3}H]TMP prepared as already described[4], by the WILZBACH procedure, was diluted with 10 parts of non-radioactive TMP and phosphorylated by the SMITH AND KHORANA[12] method.

[^{32}P]TMP was prepared from 500 µmoles TdR, 100 mC carrier free $^{32}PO_4$ and 225 mg 85 % orthophosphoric acid by the method of HURWITZ[13]. When the initial reaction was complete, the [^{32}P]TMP and unreacted TdR were separated from inorganic ortho- and pyrophosphate by adsorption on a charcoal column, 10 cm × 3.5 cm. The column was washed with water and 0.01 *M* $NaHCO_3$ (see ref. 14) until the radioactivity of the eluate dropped to a low level. The TdR and TMP were then eluted with 70 % aqueous ethanol 0.14 *M* with respect to ammonia. The eluate was concentrated *in vacuo* at 37° and the concentrate applied to a Dowex-1-Cl column. The column was washed with water to remove all traces of TdR and the TMP was eluted with 0.05 *N* HCl. The eluate was concentrated *in vacuo* at 37° and then lyophilised. Paper chromatography of the TMP showed that it consisted essentially of [^{32}P]TMP with a little contaminating [^{32}P]-inorganic orthophosphate but no thymidine, pyrophosphate or TMP-3′. The overall yield was 108 µmoles TMP. This TMP was used directly as previously described for the preparation of [^{32}P]TTP. The specific activity of the product was approximately $2 \cdot 10^6$ counts/min/*µmole* as determined in a windowless gas flow counter.

Other samples of [^{32}P]TMP were prepared by the method of TENER[15] from TdR and [^{32}P]cyanoethylphosphate. [^{32}P]dAMP, [^{32}P]dGMP and [^{32}P]dCMP were also prepared in this way. The specific activity of all these nucleotides was $0.5–2.0 \cdot 10^6$ counts/min/μmole.

DNA for use as primer was obtained from the 105,000 × *g* sediment of the osmotically disrupted Ehrlich ascites cells by the detergent procedure of KAY, SIMMONDS AND DOUNCE[16].

Fractionation of Ehrlich ascites cell extracts

Extracts of Ehrlich ascites cells prepared as described above were adjusted to pH 4.5 in the cold by the addition of 1 *N* acetic acid. The resulting suspension was centrifuged at 600 × *g* for 10 min and the clear supernatant was decanted into a separate vessel and neutralised with 1 *N* NaOH (fraction AS 4.5). This fraction contains the larger part of the kinase activities and was generally used as a source of enzyme in inhibition tests. The sediment was dispersed in ice-cold distilled water to give a protein concentration of 6–8 mg/ml and the pH was adjusted to 7.0–7.5 by the addition of 0.1 *N* NaOH (fraction AP 4.5). This fraction contains the bulk of the polymerase activity and was generally used as a test material for polymerase inhibition.

Fractionation of liver extracts

Extracts of normal and regenerating liver were fractionated by the serial addition of solid ammonium sulphate. The precipitates were centrifuged down and dissolved in a volume of water equal to the original volume of extract.

Assay of kinases

Thymidine kinase (the enzyme catalysing the formation of TMP from TdR), TMP kinase (the enzyme responsible for the formation of TDP from TMP), and TDP kinase (the enzyme responsible for the formation of TTP from TDP) were assayed as described previously[4].

Kinases responsible for the formation of dATP, dGTP and dCTP from dAMP, dGMP and dCMP were assayed as follows:

The tissue extracts were incubated for 90 min at 37° in Tris buffer pH 7.4 in the presence of KCl, $MgCl_2$ ATP and the appropriate substrate. The tubes were chilled in ice and treated with 2 *N* perchloric acid to a final concentration of 0.6 *N*.

The precipitated protein was centrifuged down and the supernatant fluid neutralised with KOH. The precipitated $KClO_4$ was removed by centrifugation and the supernatant fluid either stored in the frozen state or processed immediately.

In each experiment a non-incubated control was set up in which protein was precipitated with perchloric acid before addition of substrate.

The neutral extract was diluted to about 20 ml and applied to a Dowex-1-Cl column (6.5 × 1.5 cm). The column was washed with 20 ml water and eluted with 100-ml portions of dilute acid according to the following scheme:

[^{32}P]dCMP	1. 0.01 *N* HCl to remove monophosphates 2. 0.01 *N* HCl + 0.2 *M* LiCl to remove di- and triphosphates
[^{32}P]dAMP	1. 0.01 *N* HCl to remove monophosphates 2. 0.01 *N* HCl + 0.2 *M* LiCl to remove di- and triphosphates
[^{32}P]dGMP	1. 0.05 *N* HCl to remove monophosphates 2. 0.02 *N* HCl + 0.2 *M* LiCl to remove di- and triphosphates

The eluates were appropriately diluted for counting in an M6 liquid counter attached to a conventional scaling unit.

Polymerase assay

The enzyme responsible for the formation of polynucleotide material from dATP, dGTP, dCTP and TTP was assayed by two procedures:

Net synthesis assay: An incubation was set up containing the enzyme preparation. 100 μmole Tris buffer pH 7.9, 0.25 μmole DPN, 5 μmoles $MgCl_2$, 25 μg DNA primer previously heated to 100° for 10 min and 300 mμmoles each of dATP, dGTP, dCTP and TTP in a volume of 1 ml. Incubation was carried out at 37° in centrifuge tubes with shaking for 2 h. In this assay all tests were carried out in triplicate or quadruplicate and an equivalent number of controls to which no deoxyribonucleoside triphosphates had been added was set up. The reaction was stopped by immersing the tubes in a mixture of ethanol and solid CO_2 and the material was either processed immediately or stored at —20° until required. The frozen incubation mixtures were allowed to thaw at 0° and, where the amount of enzyme protein was very low, 0.2 ml of 1 % solution of bovine serum albumin was added to facilitate precipitation. 0.5 volume ice-cold 2.1 *N* $HClO_4$ was added to precipitate protein and nucleic acid and the mixture was centrifuged. The supernatant fluid was decanted and the precipitate was washed twice more with cold 0.7 *N* $HClO_4$ to remove soluble nucleotides. The washed precipitate was dissolved in 2 ml 0.3 *N* KOH and two 0.8-ml portions of the solution were taken for the estimation of DNA by the CERIOTTI[17] procedure. The difference in DNA content between control and test incubations was taken as a measure of new polynucleotide synthesis.

The incorporation of [^{32}P]TTP: Reaction mixtures were set up containing 25 μmoles Tris buffer pH 7.9, 62.5 mμmoles DPN, 1.25 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 50 μg DNA (previously heated for 10 min at 100° and cooled quickly) and, generally, 50 mμmoles each of dATP, dGTP, dCTP and [^{32}P]TTP. The polymerase preparation was added, the total volume adjusted to 0.25 ml with water and the tubes incubated at 37° for 2 h in a shaking incubator. After incubation, the amount of radioactivity in the DNA was determined by the method described by BOLLUM[18]. 50-μl aliquots of the incubation mixtures were pipetted on to numbered discs of Whatman No. 1 filter paper, 1 in in diameter. The discs were dropped into a beaker of cold 5 % (w/v) TCA containing 15 ml TCA per disc and 5–10 min after the last disc had been added the batch was washed by swirling in the beaker. The TCA was removed by suction and replaced by fresh TCA and the whole process repeated twice more. Finally the discs were washed with 95 % ethanol, removed, dried, and counted in a Nuclear Chicago windowless gas flow counter. The above procedure was found to remove all radioactivity from non-incubated control discs. This assay has been shown to be reliable by comparison with the net synthesis method.

For studies on inhibitory factors, 0.075 ml of polymerase preparation was incubated with an equal volume of solution of potential inhibitor.

MATERIALS

ATP, dAMP, dGMP, dCMP, TMP, TdR, deoxyadenosine, deoxyguanosine, deoxycytidine and DPN were purchased from the Sigma Chemical Corporation. Some specimens of ATP and DPN were purchased from C. F. Boehringer and of dAMP, dGMP, dCMP and TMP from Schwarz Bioresearch Inc. and from the California Corporation. [^{3}H]-TdR of specific activity 360 mC/mmole or 1.9 C/mmole was purchased from Schwarz Bioresearch Inc.

RESULTS

The results obtained with AS and AP from the supernatant fraction from Ehrlich ascites cells (Table I) show that such extracts contain an active polymerase and kinases capable of converting 75 % of the added TdR to TTP.

In extracts of normal rat liver, polymerase activity is low (Table I) but is increased in regenerating rat liver 48 h after partial hepatectomy. However, the addition of normal rat liver extracts to regenerating rat liver extracts greatly reduces the polymerase activity (Table I). The same effect is observed when rat liver extract is added to an ascites preparation (Table I).

Extracts of rabbit liver also show a low polymerase activity whereas extracts of bone marrow give a high value (Table I). Addition of liver extract to bone marrow extract reduces the polymerase activity to a very low level (Table I).

A similar picture is obtained with kinase activity (Table I) which is higher in extracts of regenerating liver, bone marrow and ascites tumour than in extracts of normal liver. Again, addition of extracts of normal liver to extracts of regenerating liver, bone marrow or ascites tumour reduces kinase activity as measured by the amount of TdR converted to phosphorylated derivatives (Table I).

TABLE I

POLYMERASE AND KINASE ACTIVITY IN EXTRACTS OF DIFFERENT TISSUES

Polymerase assay: Reaction mixtures contained 25 μmoles Tris buffer pH 7.9, 62.5 μmmoles DPN, 1.25 μmoles MgCl$_2$, 1 μmole 2-mercaptoethanol, 50 μg DNA (previously heated for 10 min at 100° and cooled quickly), generally 50 mμmoles each of dATP, dGTP, dCTP and [^{32}P]TTP and 0.075 ml of the polymerase preparation, all in a total volume of 0.25 ml. The mixtures were incubated with shaking in 10 × 1 cm test tubes for 2 h at 37°.
Kinase assay: Reaction mixtures contained 5.5 μmoles MgCl$_2$, 5.5 μmoles ATP, 110 μmoles Tris buffer pH 7.9, 0.5 μC[^{3}H]TdR and 0.35 ml of kinase preparation, all in a total volume of 1.1 ml. The mixtures were incubated with shaking in 25-ml conical flasks for 90 min at 37°. For inhibition effects, incubation mixtures contained equal volumes of enzyme and inhibitor preparations. These were 0.075 ml and 0.35 ml for polymerase and kinase respectively.
For liver and bone marrow assays whole extracts were employed.
For ascites cells, fraction AS 4.5 was used for kinase assays and fraction AP 4.5 for polymerase assays.

Tissue	*Polymerase**	*Kinases***		
		TMP	*TDP*	*TTP*
Ascites tumour	4.7	430	830	2130
Ascites tumour + rat liver	0.4	14	12	0
Rat liver	1.2	10	3	1
Regenerating rat liver	7.3	30	70	50
Normal + regenerating rat liver	0.6	40	20	7
Rabbit liver	0.4	10	2	1
Rabbit bone marrow	5.3	20	80	130
Rabbit liver + bone marrow	0.2	20	20	20

* mμmoles [^{32}P]TTP incorporated in 2 h /mg protein.
** μμmoles formed in 1.5 h /mg protein.

TABLE II

KINASE ACTIVITIES IN EXTRACTS OF EHRLICH ASCITES CELLS AND OF LIVER TISSUE

The reaction mixture (1 ml) contained 50 µmoles Tris buffer pH 7.4, 80 µmoles KCl, 25 µmoles $MgCl_2$, 25 µmoles ATP (Na salt), 5 µmoles substrate, and two of the following —0.2 ml ascites extract, 0.2 ml liver extract, 0.2 ml water. Incubation time 90 min. Temperature, 37°.

Substrate	*Material tested*	*Total*	*% applied counts recovered*	
			Monophosphate	*Diphosphate + Triphosphate*
[^{32}P]dCMP	Control	100	95	5
	Ascites	85	69	16
	Liver	85	65	20
	Ascites + liver	87	59	28
[^{32}P]dAMP	Control	100	95	5
	Ascites	87	63	18
	Liver	85	60	25
	Ascites + liver	84	48	36
[^{32}P]dGMP	Control	100	91	9
	Ascites	82	64	18
	Liver	83	61	22
	Ascites + liver	75	39	36

It should be noted that the pattern of results obtained with the kinases for dAMP, dGMP and dCMP is different from that found for the kinases of thymine derivatives. Table II shows appreciable levels of these kinases in extracts of normal liver and ascites tumour cells. The addition of extracts of normal liver to ascites cell extracts causes no inhibition of kinase activity and even results in an additive effect. Moreover the difference between extracts of normal and of regenerating liver is but slight with these substrates (Table III), although the percentage of counts recovered is consistently greater for regenerating than for normal liver.

Although normal liver extracts show low polymerase activity, they nevertheless

TABLE III

KINASE ACTIVITIES IN EXTRACTS OF NORMAL AND REGENERATING RAT LIVER*

The conditions were as described in Table II. The reaction mixture (1 ml) contained 0.4 ml liver extract.

Substrate	*Material tested*	*Total*	*% applied counts recovered*	
			Monophosphate	*Diphosphate + Triphosphate*
[^{32}P]dCMP	Control	100	92	8
	Normal liver	88	59	29
	Regenerating liver	96	65	31
[^{32}P]dAMP	Control	100	93	7
	Normal liver	65	21	48
	Regenerating liver	89	39	50
[^{32}P]dGMP	Control	100	89	11
	Normal liver	62	36	26
	Regenerating liver	88	51	37

* 48 h after partial hepatectomy.

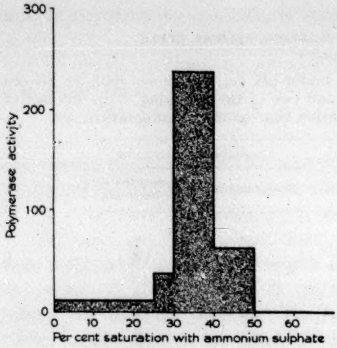

Fig. 1. Polymerase activity in fractions of normal liver extract prepared by ammonium sulphate precipitation. Enzyme activity was assayed as described in the text using 0.075 ml of each fraction after dialysis overnight against 0.1 *M* Tris buffer 0.001 *M* with respect to mercaptoethanol. Activity is expressed in terms of total counts/min of acid-precipitable radioactivity per reaction tube.

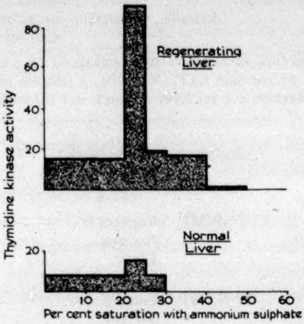

Fig. 2. Thymidine kinase activity in fractions of normal and regenerating rat-liver extracts prepared by ammonium sulphate precipitation. Enzyme activity was assayed as described in the text using 0.35 ml of a 1:5 dilution of the ammonium sulphate fractions, which had been dialysed overnight against 0.1 *M* Tris buffer 0.001 *M* with respect to mercaptoethanol. Activity is expressed as % conversion to TMP.

TABLE IV

% INHIBITION OF EHRLICH ASCITES POLYMERASE AND KINASES BY EXTRACTS OF VARIOUS TISSUES

The conditions were as described in Table I.

Tissue	*Polymerase*	*Phosphorylation to*	
		TMP + TDP + TTP	*TDP + TTP*
Rat liver	64	62	87
Rabbit liver	60	51	65
Rabbit kidney	92	0	39
Rabbit brain	0	0	0
Rabbit muscle	0	4	11
Calf serum	64	0	46
Bovine albumin	8	0	0

TABLE V

INHIBITION OF RABBIT TISSUE POLYMERASES BY LIVER AND KIDNEY EXTRACTS

Enzyme activity was assayed as described in Table I using 0.075 ml of polymerase preparation and an equal volume of liver or kidney extract.

Polymerase source	*% inhibition by*	
	Liver	*Kidney*
Spleen	23	85
Thymus	53	95
Bone marrow	85	95

contain an active enzyme which is concentrated mainly in the fraction precipitated between 30 and 40 % saturation with ammonium sulphate (Fig. 1).

The TdR kinase activity is concentrated in the fraction precipitated by ammonium sulphate between 20 and 25 % saturation and is obviously much higher in regenerating liver (Fig. 2). Such a fraction from normal liver phosphorylates 3 to 4 times as much TdR as does whole extract. No further increase in activity is obtained on subsequent fractionation with ECTEOLA columns[4].

The presence of interfering factors towards the polymerase and thymidine kinases of ascites tumour cells is shown in Table IV. Factors inhibiting polymerase are pronounced in liver, kidney and serum but not in brain, muscle or bovine serum albumin. The phosphorylation of thymidine is strongly inhibited by liver extracts but not by extracts of the other tissues studies while the formation of TDP and TTP is inhibited by liver extracts and by extracts of kidney, by serum and to a much less pronounced extent by muscle extracts (Table IV).

Similar inhibition of polymerase activity from some other sources by extracts of liver and kidney is shown in Table V.

No inhibition of polymerase or kinase activity was observed with boiled liver extract, with the acid-soluble fraction prepared from liver extract by removal of protein with acid, or with dialysates of liver extract.

DISCUSSION

The results in Table I show that extracts of liver and ascites cells are capable of phosphorylating dCMP, dGMP and dAMP. This is in agreement with the results of HECHT, POTTER AND HERBERT[19] for dCMP with normal liver homogenates and of SABLE *et al.*[20] for dAMP with rabbit kidney mitochrondria. It is clear, however, from Table I that the addition of liver extract to ascites extract results in no inhibition of phosphorylation and indeed shows an additive effect.

Extracts of regenerating liver also phosphorylate dCMP, dGMP and dAMP (Table III) in agreement with the results of CANELLAKIS AND MANTSAVINOS[8, 21] and of BOLLUM AND POTTER[6]. Our results show that there is no great difference between extracts of normal and of regenerating liver.

The position as regards the phosphorylation of thymidine and thymidine nucleotides is quite different (Table I). Extracts of rapidly proliferating cells such as ascites tumour, regenerating rat liver and rabbit bone marrow catalyse extensive formation of TMP, TDP and TTP from thymidine whereas extracts of non-proliferating tissues[4] such as normal liver have little activity. The development of enzymes for the phosphorylation of thymidine derivatives in regenerating rat liver has already been recorded[4, 6-9, 21, 22].

The results shown in Table I, however, make it clear that the addition of normal liver extract to the systems derived from ascites cells, regenerating rat liver or rabbit bone marrow causes a pronounced diminution both in the phosphorylation of thymidine and in polymerase activity. Such an inhibitory effect is also found with extracts of kidney and with serum but not with extracts of other tissues such as brain.

The fact that the inhibitory action of normal liver extracts is confined to the thymidylate system and is not observed with the kinases for dCMP, dGMP and dAMP focuses attention on the control of the formation of TTP in the regulation of

DNA biosynthesis. This is in agreement with the views of POTTER[23, 24] and of CANELLAKIS *et al.*[22].

The fact that the activity of TdR kinase in extracts of normal liver is increased on ammonium sulphate fractionation suggests that the low activity of the enzyme in unfractionated extracts is due in part at least to the presence of interfering factors.

Although tissue extracts which reduce phosphorylation of TdR also inhibit the polymerase (Table I, IV, V) the inhibition of the two systems is not always in parallel (Table IV). This suggests that different factors are involved.

While the exact nature of these inhibitory factors is still under investigation, it is already clear that the effect is not due to simple dephosphorylation of the substrates or products[10].

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STUDIES ON THE MECHANISM REGULATING PERIODIC DNA SYNTHESIS IN SYNCHRONIZED CULTURES OF *ALCALIGENES FECALIS*

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SUMMARY

Addition of deoxycytidine, deoxyguanosine, deoxyadenosine, and thymidine (10^{-4} *M* each) to the growth medium has been demonstrated to destroy periodic stepwise DNA synthesis in synchronous cultures. All four deoxyribosides are necessary. The effect is observed only if the deoxyribosides are added during DNA synthesis. It is accompanied by a loss of periodicity of RNA synthesis and of cell division.

At high medium concentrations (greater than 10^{-3} *M*), deoxyadenosine has been shown to selectively block DNA synthesis. This effect may be reversed by adenosine.

When a step of DNA synthesis—in a synchronous culture—is blocked, reversal of the inhibition results in resumption of DNA synthesis at that time when a second synthetic step occurs in cultures not treated with deoxyadenosine. This is true regardless of the time at which inhibition is initiated or reversed. This has been taken to suggest that DNA synthesis occurs only during certain "permitted" portions of the division cycle.

A hypothetical mechanism for controlling periodic DNA synthesis is discussed.

INTRODUCTION

Cultures of *Alcaligenes fecalis LB* in which the individual cell division cycles have been synchronized, show stepwise periodic synthesis of DNA and of a sedimentable RNA fraction[1]. In addition, the content of acid-soluble phosphorus within the cell changes in cycles whose period is the same as that found for nucleic acid synthesis and cell division[2]. Certain biological properties, such as the ability of the cell to concentrate intracellular metabolites and its susceptibility to penicillin, increase or decrease periodically[3,4]. On the other hand, synthesis of either cytoplasmic or cell wall protein is not periodic[3].

Several different systems for synchronizing bacteria have been described during the past few years[5]. In some of these[6,7] synthetic events occur in cycles, as in *Alcaligenes fecalis*; in others this is not the case[8,9]. Recent studies on DNA synthesis in single bacterial cells taken from exponential cultures have revealed, a lack of periodi-

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

city during their division cycle[10]. This lack of periodicity in single cells was shown to exist in the same organism which had exhibited stepwise DNA synthesis in synchronized cultures[6]. As such data have accumulated, it has become evident that bacteria—unlike cells of metazoan and some protozoan systems—do not display periodic synthesis of DNA as a necessary characteristic of the division cycle.

Cells of plant, animal, or protozoan origin show pronounced variations in the rates at which different compounds are synthesized during the division cycle. Regulation of the time of occurrence of intracellular events appears to be coordinated in a precise order[11-14].

What is the nature of the timing device which controls this coordination?

The demonstrated periodicity of several biochemically measurable parameters in the division cycle of *Alcaligenes fecalis* suggests this organism as a useful model for experimental approaches to this question.

In this investigation we have studied the control of periodic DNA synthesis. DNA was chosen because its periodicity could be easily observed and did not appear likely to be merely a reflection of some other periodic process within the cell. Previous studies had demonstrated that this synthetic periodicity was independent of both cytokinesis and gross protein synthesis[15].

Investigations of several systems have indicated that precursors of DNA may play an important role in the regulation of its synthesis. Studies on lily anther, on bacteria, and on tetrahymena have shown that deoxynucleosides or -tides accumulate prior to nuclear or cellular division[16-18]. Cells of *S. typhimurium* grown at different temperatures contain different quantities of acid soluble deoxyribosidic compounds. Upon shifting growing cells from a temperature of 25 to 37°, a sudden synthesis of DNA takes place which ceases as the quantity of these compounds is reduced[17]. Accumulation again occurs prior to the resumption of DNA synthesis.

In the present study, the addition of deoxyribosides to the growth medium has been used to interfere with periodic DNA synthesis. The results have made it clear that regulation of periodicity can occur at the precursor level.

MATERIALS AND METHODS

The methods for obtaining synchronized growth of *Alcaligenes fecalis LB* in a casamino acid medium have been described[3]. The methods used in the estimation of turbidity and cell number by the Coulter particle counter are also the same as previously published[3]. Measurement of DNA and RNA were carried out, as before, by the diphenylamine and orcinol reactions, respectively,[19,20]. Protein was estimated by the modified Folin reaction[21].

Samples for nucleic acid analysis were frozen until analyzed. Preparation of such samples for analysis followed the procedure already described[1] with the exception that samples from cultures treated with deoxynucleosides, particularly deoxyadenosine or -guanosine, were given three extra washes with cold 5 % TCA to remove all traces of deoxyribosides in the medium. In the majority of experiments deoxyadenosine was used as a comparative color standard for the diphenylamine reaction to obtain relative DNA values. Adenosine was used for the orcinol reaction. In obtaining the data given in Table I, standard sources of DNA and RNA were used to obtain absolute values.

Deoxyribosides used in these studies were obtained from the Sigma Chemical Company and were found to be chromatographically pure.

RESULTS

The DNA of *Alcaligenes fecalis* contains thymidine, deoxyguanosine, deoxyadenosine and deoxycytidine. When these deoxyribosides (10^{-4} *M*) are added to a synchronized culture, periodic synthesis of DNA is lost. This effect is shown in Fig. 1.

Other experiments have shown that the presence of *all four deoxyribosides* is necessary to achieve loss of periodicity. With the omission of any single deoxyriboside from the mixture, periodic DNA synthesis continues.

Figs. 2A and B illustrate the effect of adding deoxyribosides to a synchronous culture at different intervals. In the experiments in Fig. 2A samples of a synchronized culture were removed at 5, 10, 20, and 35 min and deoxyribosides added as in the previous experiment. DNA synthesis was followed in the control and in the four experimental aliquots of which only two showed a loss of periodicity. Those samples to which deoxyribosides were added before or after periods of rapid DNA synthesis were not affected. In sample 1 (deoxyribosides added before DNA synthesis), the

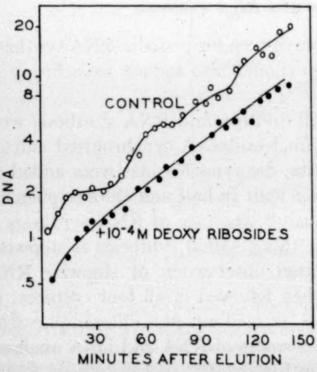

Fig. 1. Loss of periodic DNA synthesis upon addition of deoxyribosides. Deoxyadenosine, deoxyguanosine, thymidine, and deoxycytidine were added to a synchronized culture at 2 min after the experiment was begun. Each was added at a concentration of 10^{-4} *M*. Ordinates: DNA in relative units. Ordinate values for the curve with deoxyribosides were divided by 2.2 for clarity of presentation. Abscissa: Minutes after initiation of experiment by elution from filter paper. For the absolute quantities of DNA, RNA, and protein found in *Alcaligenes fecalis*, see Table I.

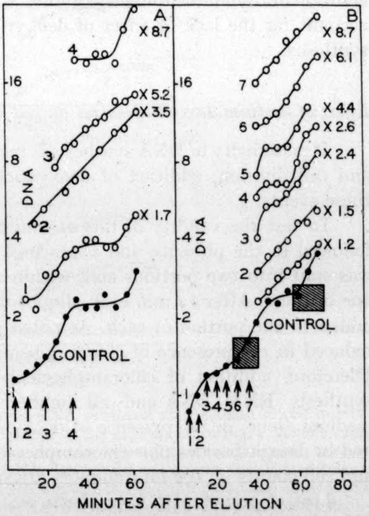

Fig. 2. Effect of adding deoxyribosides to a synchronized culture of *Alcaligenes fecalis* at different times. Times of addition of deoxyribosides are indicated by the arrows. Aliquots containing deoxyriboside have been multiplied by an arbitrary factor to enhance clarity of presentation. This factor is given at the right of each graph. Ordinates: DNA in relative units. Abscissa: Minutes after elution. The shaded area in (B) represents periods of cell division (cytokinesis).

continued presence of added deoxynucleosides did not lead to either an early burst of DNA synthesis or eventual loss of periodicity. Thus, there appears to be a crucial time at which deoxyribosides must be added to result in loss of periodicity. If added before this time, their continued presence does not exert an effect. This may be seen more clearly in Fig. 2B where deoxyribosides were added to a synchronous culture at different times during the first cycle of synthesis, and the effect on the second cycle was studied. Addition of deoxyribosides was effective only when it occurred at a time of DNA synthesis. Thus, periodicity was lost only in samples 1, 2, and 7 and partially disappeared in both 3 and 6. On the other hand, both samples 4 and 5 retained periodicity.

Control experiments have shown that cellular uptake of deoxyribosides does not significantly reduce their medium concentration during the course of the experiment. This is true regardless of the time at which they are added during the first cycle of DNA synthesis. Thus, the presence of deoxyribosides in the medium prior to the period of DNA synthesis results in a change in cell metabolism such that the cell is no longer affected by their presence during subsequent synthesis. A phosphorylase that splits thymidine to thymine plus deoxyribosephosphate has been described in *E. coli*[22]. Recent evidence has indicated that it may be induced within 10 min after the addition of thymidine to the medium[23]. A similar adaptation in *Alcaligenes fecalis* may account for the lack of effect of deoxyribosides added prior to the period of DNA synthesis.

Effect of medium deoxynucleosides on cell division and RNA synthesis

If periodicity in DNA synthesis is responsible in turn for periodic RNA synthesis and cell division, addition of deoxynucleosides should also induce asynchrony in these events.

To test the validity of this assumption, cell division and RNA synthesis were followed in the presence and absence of deoxynucleosides. A synchronized culture was split into two portions and, within a minute, deoxynucleosides were added to one of them. After 12 min each aliquot was again split in half and chloramphenicol added to one portion of each. As noted previously[15], the rate of RNA synthesis is reduced in the presence of chloramphenicol but this residual synthesis is stepwise. Therefore, addition of chloramphenicol facilitated observation of stepwise RNA synthesis. RNA, DNA and cell number were then followed in all four cultures: in medium alone, in the presence of deoxyribosides, in medium plus chloramphenicol, and in deoxyribosides plus chloramphenicol. The results of RNA and DNA analyses and cell counts on the four aliquots are shown in Fig. 3.

Loss of periodic DNA synthesis was accompanied by loss of synchrony in cell division (cytokinesis). In addition, the pattern of RNA synthesis was changed. Most striking in this respect was the increase in the early rate of RNA synthesis, both in the presence and absence of chloramphenicol. In both cases, the pattern of RNA synthesis clearly followed that of DNA synthesis. Loss of periodic DNA synthesis resulted in an increase in the amount of DNA in the culture during periods in which DNA synthesis ceased in the control cultures (see dotted lines in Fig. 3). Thus, an increase in the content of DNA appeared to result in an increased rate of RNA synthesis.

Inhibition of DNA synthesis by high concentrations of deoxyadenosine

In the course of experiments employing deoxyribosides at high concentrations, it was found that cells ceased to grow properly in the presence of deoxyadenosine in excess of 10^{-3} *M*. Cells developed into long filamentous forms and eventually ceased dividing. This effect was found only with deoxyadenosine and could be reversed by adenosine. Analysis of cells treated with deoxyadenosine revealed that a primary effect of this compound was on DNA synthesis. The nucleic acid composition of such cells is shown in Table I. Cells grown in deoxyadenosine plus adenosine were essentially identical in composition with those grown in plain medium, while those grown in deoxyadenosine alone had an abnormally low DNA content relative to RNA or protein. This finding indicated that deoxyadenosine treatment selectively blocked cell division and DNA synthesis, leading to a *larger* cell with relatively more RNA and protein than DNA. A similar effect has been described for ascites tumor cells[24].

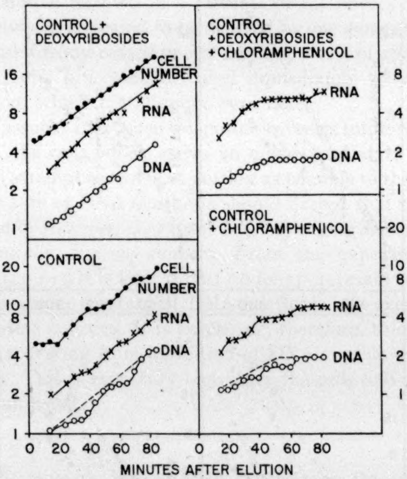

Fig. 3. Effect of loss of periodic DNA synthesis on RNA synthesis and increase in cell number. Deoxyribosides were added to a synchronized culture as in the experiment in Fig. 1. After 12 min, both the control and the deoxyriboside containing cultures were split in two portions, to one of which chloramphenicol was added. Values for DNA, RNA, and cell number in all four cultures are directly comparable. The dotted lines in the DNA graph in the control and the control-plus-chloroamphenicol cultures represent the course of DNA synthesis after the addition of deoxyribosides. Ordinates: DNA, RNA, and cell number in arbitrary units. Abscissa: Minutes after initiation of synchrony.

The time course of DNA inhibition by deoxyadenosine and the reversal of this inhibition by adenosine were studied in the following experiment. Deoxyadenosine was added to an exponentially growing culture at a concentration of $1.25 \cdot 10^{-3}$ *M* to initiate the experiment. At different intervals thereafter, samples were removed for analysis or cell count. At 40 (○), 120 (×), and 200 (△) min after the addition of

TABLE I

EFFECT OF DEOXYADENOSINE ON THE CHEMICAL COMPOSITION OF *Alcaligenes fecalis*

100 ml cultures were grown for 2 h in $1.25 \cdot 10^{-3}$ *M* deoxyadenosine (DOA), $1.25 \cdot 10^{-3}$ *M* deoxyadenosine plus $1.25 \cdot 10^{-3}$ *M* adenosine (DOA + A), and in normal medium (C). At the end of this time the average volumes per cell were: (DOA), 1.14 μ^3; (DOA + A), 0.67 μ^3; (C), 0.69 μ^3. Total cell numbers per 100 ml were: (DOA), $2.04 \cdot 10^{10}$ ml; (DOA + A), $3.84 \cdot 10^{10}$; (C), $4.56 \cdot 10^{10}$. Protein is given in equivalents of bovine serum albumin (Armour), RNA in equivalents of ribonucleate (California Foundation), DNA in equivalents of deoxyribonucleate (California Foundation).

	DOA 10^{-14} *g*	*DOA + A* 10^{-14} *g*	*C* 10^{-14} *g*
DNA per Cell	1.48	1.23	1.46
RNA per Cell	22.5	8.92	9.02
Protein per Cell	33.4	12.5	18.4

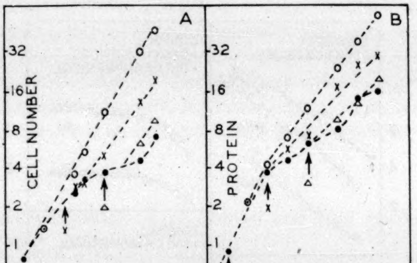

Fig. 4. Effect of deoxyadenosine on RNA, DNA, and protein synthesis and on increase in cell number. Deoxyadenosine was added at 0 min to an exponential culture at a concentration of $1.2 \cdot 10^{-3}$ *M*. Aliquots of the culture were diluted at intervals into adenosine. The intervals were 40 min (○), 120 min (×), and 200 min (△). Increase in cell number, protein, DNA, and RNA was followed in the original culture and in the aliquots to which adenosine was added. Ordinates: Cell number, protein, DNA, and RNA in arbitrary units. Abscissa: Minutes after adding deoxyadenosine.

deoxyadenosine, aliquots of the culture were diluted 3-fold into 10^{-3} *M* adenosine and these daughter cultures were also analyzed at intervals. The results are shown in Fig. 4.

For a short period after addition of deoxyadenosine, cell number and the amount of nucleic acids and protein continued to increase at the normal rate (*i.e.*, 40 min for a 2-fold increase). Subsequently, synthesis or division became slower and even ceased. After a further interval, the cells appeared to adapt themselves to the presence of deoxyadenosine and once again synthetic rates increased. Cell division and DNA synthesis were inhibited earlier than RNA or protein synthesis leading to cells with relatively high RNA and protein contents. After this occurred, all synthetic parameters appeared to behave similarly, although cell division remained comparatively retarded. As a result, cells became progressively larger although they preserved their new RNA: protein: DNA ratios. Such cells were viable and, indeed, cells will retain their viability in concentrations of deoxyadenosine as high as 10^{-2} *M*.

Addition of adenosine resulted in a reversal of the effects of deoxyadenosine. Resumption of cell division appeared to be delayed by one generation period in those instances where it had already ceased or was in the process of stopping. In contrast, the rates of all synthetic processes increased immediately relative to the deoxyadenosine culture from which the aliquots were taken.

It is natural to assume that when adenosine reverses inhibition of RNA, DNA, or protein synthesis, the cells which are in an unbalanced state (more protein and RNA than DNA) will attempt to revert as quickly as possible to their normal composition. To do this, the rate of DNA synthesis should exceed that of RNA or protein. This is what was found. *However, the rate of DNA synthesis did not exceed that found normally in exponentially growing cultures.* From the experiments[1] on stepwise synthesis of DNA (Figs. 1–3) it is known that under appropriate conditions (during a synthetic step for example) the rate of DNA synthesis can exceed the rate found in exponentially growing cultures. It is surprising, therefore, that in the experiment just described, cells recovering from inhibition of DNA synthesis did not synthesize DNA at a faster rate. This is especially true since the cells had a higher content of RNA and protein than usual.

The temporal control of DNA synthesis

Limitation of the recovery rate of DNA synthesis in the experiment in Fig. 4 would occur if DNA synthesis by individual cells was prohibited for certain periods. In such a case, periods in which synthesis of DNA was allowed would occur at random in an asynchronous culture as do the division cycles of individual cells. Temporal control of this process could be independent of the synthesis of DNA. Thus, upon adding adenosine to deoxyadenosine-inhibited cells, synthesis of DNA would be free to resume in each cell *as soon as an allowed period occurred*. In some cells this would be immediately; others could prohibit synthesis for as long a time as is *normally* required for a complete division cycle.

It was possible to test this hypothesis with synchronous cultures. Fig. 5 gives the results of an experiment in which a step of DNA synthesis was blocked, the block removed at different times, and the pattern of DNA synthesis measured. Deoxyadenosine was added to a synchronous culture at 20 min at a concentration of 10^{-2} *M*. This concentration of deoxyadenosine blocked protein and RNA synthesis as well as DNA synthesis. Turbidity was used to estimate increases in mass, since the small amount of material available precluded reproducible direct analyses of protein. As may be seen in Fig. 5, synthesis of DNA and RNA, as well as increase in total mass, ceased following addition of $10^{-2}\,M$ deoxyadenosine. On the other hand, the increase in cell number continued, indicating that cytokinesis could proceed.

At 35 and 50 min, 15 and 30 min after the addition of deoxyadenosine, aliquots of the culture were diluted 6-fold out of deoxyadenosine into $1.5 \cdot 10^{-3}\,M$ adenosine. *Following reversal of the deoxyadenosine block, DNA synthesis did not occur until 65 min, regardless of when the block was reversed. This is the time at which a step of DNA synthesis occurred in the untreated synchronous culture.* In contrast, cytokinesis was delayed by a period equivalent to that for which the cells were in deoxyadenosine.

RNA synthesis commenced immediately upon reversal of the block but also showed periodicity which was independent of the time of addition of adenosine. Turbidity showed a behavior somewhat similar to that of DNA, although such a stepwise increase in mass was not found in synchronized cultures which had not been treated with deoxyadenosine.

The pattern of DNA synthesis shown in Fig. 5 was obtained in a large number

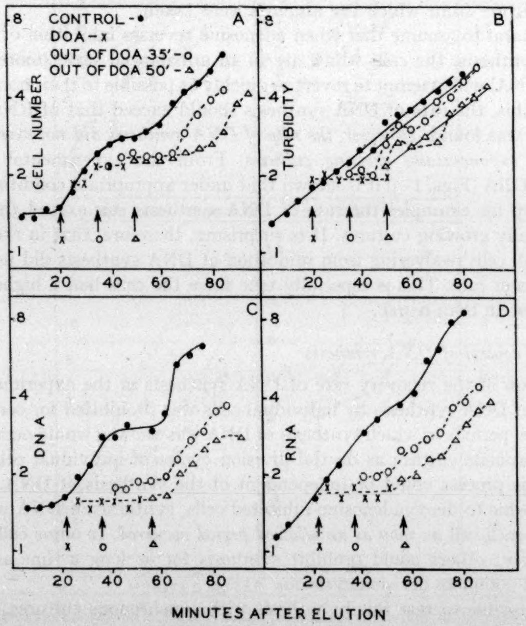

Fig. 5. Interruption of DNA synthesis by deoxyadenosine and the pattern of synthesis following removal of inhibition. Deoxyadenosine was added to the synchronized culture at 20 min at a concentration of $10^{-2}\,M$ (×). The culture was diluted out of deoxyadenosine into adenosine at 35 (○) and 50 (△) min. Cell number, turbidity, DNA, and RNA were measured. Ordinates: Cell number, turbidity, DNA, and RNA in arbitrary units. Abscissa: Minutes after elution.

of experiments and was the same when deoxyadenosine was added 20 min before the synthetic step, or when its effect was reversed at times other than 35 or 50 min. When the effect of deoxyadenosine was reversed during a period in which synthesis was taking place in the control (untreated) culture, residual synthesis occurred immediately. This has been seen repeatedly in experiments such as that shown in Fig. 5, *i.e.*, when deoxyadenosine was diluted at 35 min, a small burst (10%) of synthesis took place immediately. This did not happen in experiments where deoxyadenosine was diluted at 40 or 45 min.

When $10^{-2}\,M$ deoxyadenosine was added to an exponential, asynchronous culture, synthetic processes resumed immediately at the normal exponential rate following addition of adenosine. No periodicity was observed. However, a lag occurred before resumption of cell division.

From the data in Fig. 5 we can conclude that the periods between DNA synthetic steps are timed independently of the quantity of DNA per cell or of the occurrence of a previous burst of DNA synthesis. The data suggest that the proposed hypothesis of "allowed periods" of DNA synthesis is correct.

DISCUSSION

A discussion of the data may be conveniently divided into three portions:
(a) The relationship of RNA and DNA synthesis to the cell division cycle.
(b) Properties of the mechanism controlling the periodicity of DNA synthesis.
(c) Models of periodic control which are consistent with the data.

(a) *Relationship of RNA and DNA synthesis to the cell division cycle*

Previous experiments had indicated that synthesis of DNA occurs at about the same time as cell division. As more experiments have been carried out (over fifty during the past year) it has become apparent that DNA synthesis in synchronous cultures of *Alcaligenes fecalis* probably takes place immediately after cell separation. This conclusion is based on the observation that DNA synthesis frequently commences 5–10 min after cell separation (Fig. 3). This difference cannot always be seen, since an increase in cell number is often delayed because cells tend to stick together. This circumstance makes it difficult to pinpoint, with absolute certainly, a sequence of events relative to cell division.

RNA synthesis has been shown to be most rapid during and immediately following the period of DNA synthesis[1]. The division cycle of cells taken from synchronized cultures of *A. fecalis* may then be pictured (with the reservations mentioned above) as cytokinesis closely followed by DNA synthesis and rapid RNA synthesis.

In experiments in which synchronized DNA synthesis is deliberately destroyed, such as that shown in Fig. 3, synchrony of cytokinesis is invariably reduced. In the majority of cases, as in Fig. 3, it is almost completely absent.

This is consistent with the hypothesis that periodic increase in cell number reflects DNA synthesis and could be caused by the latter. However, it must be remembered that cytokinesis will also depend on other cell processes which may, under certain conditions, be limiting. An example of this is the delay caused by different periods of incubation in deoxyadenosine (Fig. 5) which blocks protein and RNA synthesis as well as DNA synthesis.

Periodicity of RNA synthesis is also lost when periodicity of DNA synthesis is destroyed. An explanation of this is suggested by the increase—upon adding deoxyriboside—in the rate of RNA synthesis in the presence or absence of chloramphenicol. Since loss of periodicity of DNA synthesis is characterized by an increase in the average content of DNA of the culture during the period in which DNA synthesis would normally be retarded, increases in DNA content may be responsible for increases in the rate of RNA synthesis.

This would explain the periodic synthesis of RNA which is normally observed in synchronous cultures[1]. Thus, every time a step of DNA synthesis occurs, RNA is synthesized more rapidly.

These data support the hypothesis that periodic DNA synthesis is basic to the cell and may be responsible for the periodicity of RNA synthesis or of cell division.

(b) *What are the proporties of the mechanism controlling periodicity of DNA synthesis?*

(α) The regulatory mechanism is independent of the synthesis of DNA itself, *i.e.*, the synthesis is not autoregulatory. This follows from the experiment in Fig. 5 in which resumption of DNA synthesis was independent of the lack of a previous step of synthesis or of the period for which this synthesis was blocked. Since cytokinesis proceeded, these cells contained less DNA than normal. This finding implies that direct feedback or repression of DNA synthesis is not the mechanism whereby periodic synthesis is controlled, *i.e.*, net DNA synthesis is not limited solely by the amount of DNA per cell.

(β) Regulation of periodic synthesis can be controlled at the precursor level, since addition of deoxyribosides will destroy periodicity (Fig. 1). In the experiments described, all four deoxyribosides were required to achieve an effect. This implies physiological interference with DNA synthesis.

(γ) Destruction of periodicity at the precursor level only occurs during the period of DNA synthesis (Figs. 2A and B). Thus, it is only at this time that the regulatory mechanism becomes coupled with, or dependent on, DNA precursors. Precursors thus interfere with cessation or limitation of DNA synthesis rather than its onset or "trigger", since addition of precursors prior to DNA synthesis neither "triggers" the synthesis nor delays its onset.

(δ) the cell can adapt itself to the presence of added deoxyribosides in such a manner that the regulatory mechanism is not upset by their presence during DNA synthesis (Fig. 2B, Curves 4 and 5). This would indicate that mechanisms exist for either controlling the size of the intracellular pool or for bypassing it altogether. However, the cell requires advance warning to set up these mechanisms. As mentioned above, induction of specific enzymes which destroy deoxyribosides may play such a role*.

(ε) Following addition of deoxyribosides, periodicity of cell division is also lost. Since cytokinesis is a discontinuous event, this suggests that deoxyribosides are exerting their effect by randomizing the period between DNA synthesis in individual cells rather than by stimulating the continuous synthesis of DNA in all cells. This is only suggested by the data but appears to be a reasonable hypothesis which can be checked by autoradiographic techniques.

(c) *What models of periodic DNA synthesis can account for the data presented?*

Work in this laboratory has demonstrated that prior to a step of DNA synthesis deoxyriboside containing materials are accumulated within the cell[17]. This increase is sudden and precisely timed indicating that it may be considered a discrete event. However, the amounts of acid soluble material involved are too small to be considered as a supply of precursor material for use in DNA synthesis. This finding indicated that the sudden appearance of precursor material may be catalytically involved in the initiation of DNA synthesis. Whether or not this is correct, the findings presented in this paper make it evident that the timing of DNA synthesis can also be controlled by the presence of deoxyribosides during DNA synthesis. As we have seen, neither the amount of DNA per cell nor the lack of consummation of its synthesis are important for the functioning of the timing mechanism.

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* See NOTE ADDED IN PROOF.

What occurs during the period in which DNA may be synthesized which makes the timing mechanism so vulnerable to outside interference?

During replication of DNA, the molecule must uncoil to a sufficient extent to allow replication of sister strands. As a working hypothesis let us imagine that it is this separation of the DNA molecule which times the steps of DNA synthesis. (Such uncoiling and recoiling could even occur during deoxyadenosine inhibition.) Deoxyribosides could pair with bases on the separated strands, preventing recoiling and thus leading to additional DNA synthesis during the period in which strands are separated. Eventually the separated strands will again become intertwined and the cell will enter a period of no DNA synthesis.

Since, at best, a step of DNA synthesis is not perfectly synchronized in all cells and takes some time to accomplish, it is not unreasonable to expect that addition of deoxyribosides during a period of DNA synthesis catches individual cells in different stages of synthesis. This could prolong the synthetic period unequally for different cells. Once this has occurred, individual cells will be on different time schedules for DNA synthesis, leading to random total synthesis by the culture.

This hypothesis requires that the physical state of DNA has a profound influence on the timing of its synthesis. It demands that, in any cell, a certain period must elapse between the return of DNA to the native state and its ability to again enter the primer state.

It should be possible to test this hypothesis experimentally by means of autoradiography of single cells and by the study of the effect of deoxyribosides on the reversible denaturation of DNA.

NOTE ADDED IN PROOF

Since this paper was submitted for publication, evidence has been obtained in this laboratory that immediately upon addition of deoxynucleosides to the medium they appear in the intracellular pool, but disappear within 15 min further incubation.

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STUDIES ON s-RNA SYNTHESIS

II. ASSAY AND METHOD OF PURIFICATION OF s-RNA α, β AND γ

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SUMMARY

Methods are described for the assay and for the purification of components of the enzymic system from rat liver which incorporates ribonucleotide units derived from ribonucleoside triphosphates into a soluble form of ribonucleic acid.

INTRODUCTION

The soluble cytoplasmic fraction of rat liver contains "enzyme systems"* which incorporate ribonucleotide units derived from ribonucleoside triphosphates into the s-RNA of the same cell fraction[1-4]. During purification these enzyme systems are fractionated with the s-RNA into three distinct ribonucleoprotein components designated ribonucleoproteins α, β and γ**. Further purification of these ribonucleoproteins results in the separation of the protein component from the s-RNA component. Neither component exhibits enzymic activity when assayed alone, but full activity is restored when the protein and nucleic acid fractions are combined[5]. In the present communication the method of fractionation of these components, as well as the details of the methods of assay, are presented. Fractionation of the individual components is in progress and will be presented at a later date.

METHODS

Assay

General principle: The assay is based on the general reaction:

$$\text{XTP}^* + \text{s-RNA} \rightarrow \text{XMP-s-RNA} + \text{PP}$$

Abbreviations: s-RNA, soluble ribonucleic acid; DEAE, diethyl amino ethyl; ATP, adenosine triphosphate.

* In order to demonstrate full activity in the reaction under study, both the "protein" and the "RNA" components are required. For simplicity of communication, therefore, we wish to refer to the "protein" component as the enzyme fraction or system and to the "RNA" component as the "s-RNA" or the "RNA" component. Whether this, in effect, is the relationship of the two fractions will be discovered with further understanding of the reaction under study. Since the reactions performed by these fractions are not limited to the incorporation of ribonucleotide units into terminal positions of the RNA, we wish to avoid naming them in more specific terms until they have been defined with greater precision.

** See NOTE ADDED IN PROOF.

where XTP* is a radioactive ribonucleoside triphosphate and XMP is the corresponding radioactive ribonucleoside monophosphate. The total amount of radioactivity incorporated into acid-precipitable material is used as a measure of the extent of reaction. The reaction is stopped by the addition of perchloric acid. A small amount of carrier-protein and s-RNA is added to ensure complete precipitation of the acid-insoluble components in the incubation mixture. Adsorbed acid-soluble radioactivity is removed and the residual acid-insoluble radioactivity is determined.

Procedure

Of the protein of the original extracts (crude material from steps 1–3), 10 to 20 mg should be used per assay tube. The more purified ribonucleoprotein components should be diluted to give an optical density of 20 at 260 mμ, 1 cm light path. The standard incubation mixture consists of 0.8 ml of 0.08 *M* potassium phosphate, pH 7.2, 0.2 ml of the protein fraction (dialyzed against the same buffer), 2 μmoles $MgCl_2$ and 10 mμmoles of the XTP*. Incubation is for 10 min at 37°. The assay tubes are chilled in ice, 0.5 ml of 1.5 *N* $HClO_4$ is added, and this is followed by 0.05 ml of carrier (obtained from step 3 of this procedure). The addition of carrier is unnecessary when material isolated up to step 3 is assayed. After standing in ice for 5 min, the samples are centrifuged. The residue is washed three times with 8 ml of cold 0.2 *N* $HClO_4$. The removal of adsorbed radioactivity may be performed by one of the following two methods.

1. After the last centrifugation the perchloric acid is carefully drained from the residue; the latter is then suspended in 1 ml of 1 *M* $Na(NH_4)HPO_4$ and incubated at 37° for 5 min. The samples are chilled in ice, 0.20 ml of concentrated perchloric acid and 8 ml of 0.2 *N* cold perchloric acid are added. The perchloric acid wash is repeated once more, the excess acid is carefully drained off, 0.5 ml of 5 *N* NH_4OH added and the solution plated on a planchet, dried at 80° and the radioactivity determined.

2. The residue is washed once with 95 % ethanol and the tubes are carefully drained. After the addition of 2.0 ml of 10 % NaCl, the mixture is neutralized using phenol red as an internal indicator and heated for 30 min at 100°. The samples are chilled in ice and centrifuged. The residue is washed once with 0.5 ml of 10 % NaCl. To the combined supernatant fluid and wash, 2.5 volumes of cold 95 % ethanol are added. After standing for 2 h at —20°, the samples are centrifuged and the residue is washed once with 95 % ethanol. The tubes are drained by inversion on a paper towel. The sediments are dissolved in 0.5 ml of water, plated on planchets, dried and the radioactivity determined.

Enzyme fractionation

A summary of the purification method, with the pertinent data, is presented in Table I.

Step 1. Preparation of the rat liver extract: The method is described for approx. 130 g of rat liver. This amount of tissue (obtained from 9 rats weighing 450–500 g each), when blended, fills completely the centrifuge cups of a Spinco Model L Preparative Ultracentrifuge, Head No. 30.

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* In the crude extracts, 5 to 10 times this amount of XTP* should be used because of the extensive degradative reactions which occur. In addition, an ATP-generating system consisting of phosphoenolpyruvate and pyruvate kinase or of creatine kinase phosphate and créatine kinase should be added.

The livers of the rats are excised, chilled in ice, minced with scissors and homogenized in 2.5 volumes of a sucrose–$MgCl_2$–KCl solution (0.35 *M*–0.004 *M*–0.025 *M*) for 30 sec in a Waring blendor at top speed. The homogenate is then centrifuged for 1 h at 85,000 × *g*. The supernatant liquid is decanted and diluted with one volume of cold water.

Step 2. Ammonium sulfate fractionation: Solid ammonium sulfate is added to the level of 55 % saturation over a period of 10 min to the solution of *Step 1*. This is then allowed to equilibrate with stirring during a period of 30 min and centrifuged at 15,000 × *g* for 10 min. To the supernatant fraction, solid ammonium sulfate is again slowly added up to 85 % saturation. This suspension is centrifuged after 30 min of equilibration and the 55–85 % ammonium sulfate precipitate saved. This precipitate, which contains 80–90 % of the total enzyme activity of the initial liver extract, is suspended in 0.08 *M* potassium phosphate buffer, pH 7.2, to give a final volume of 100 ml and dialyzed overnight against two 4-l changes of the same buffer.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

	*Specific activity**	*Yield* %	*RNA*** *mg*	*mg Protein****/*mg RNA*
Soluble cytoplasm	5–10	100	45	100
Ammonium sulfate (0.55–0.85)	30–50	80	23	60
pH 5-precipitate	100–150	60	12	15
Hydroxylapatite	500–750	50	10	1
DEAE-cellulose				
"protein"	750	50	—	100
"RNA"	—	—	8–10	0.05

[*] The specific activity is expressed as the number of micromicro-moles of $[^{14}C]$AMP incorporated into s-RNA, with 1.0 mg protein, in 6 min at 25°.

[**] RNA was determined by an orcinol method[4].

[***] Protein was determined by the procedure of LOWRY[7].

Step 3. Precipitation at pH 5.2: The 55–85 % ammonium sulfate fraction is brought to pH 5.2 with 2 *N* acetic acid with constant stirring at 0°. After an equilibration time of 10 min this suspension is centrifuged, the precipitate is suspended in 20 ml of 0.08 *M* potassium phosphate buffer pH 7.2. and dialyzed overnight against 4 l of 0.02 *M* potassium phosphate, pH 7.2. This material contains 20 % of the protein, 60 to 80 % of the RNA and 80 % of the activity present in the 55–85 % ammonium sulfate fraction. The E_{260}:E_{280} is approximately 1.3 indicating a protein to RNA ratio of approximately 10:1.

Step 4. Fractionation on an hydroxylapatite column[6]*:* The dialyzed solution of *Step 3*, is diluted to 200 ml and added to a hydroxylapatite column, 20 × 2.5 cm, which has been equilibrated previously with 0.001 *M* potassium phosphate, pH 7.2. If the hydroxylapatite has been properly graded, a flow rate of 2 to 4 drops per minute is possible without the application of pressure. The elution of the enzymically active fractions may be carried out in one of two ways.

Stepwise procedure: This procedure yields three distinct ribonucleoprotein peaks designated ribonucleoproteins α, β and γ in their order of elution from this column.

These are eluted at the salt concentrations indicated in Fig. 1. The material in the peak tubes has ratios of $E_{260}:E_{280}$ of 1.6 to 1.9, which indicate a high nucleic acid content.

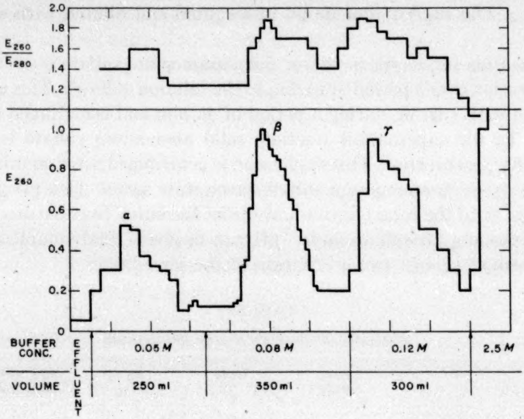

Fig. 1. Stepwise procedure for fractionation of s-RNA α, β and γ on an hydroxylapatite column.

Linear gradient elution. The linear gradient is performed with 300 ml of 0.01 M potassium phosphate, pH 7.2, in the mixer and 300 ml of 0.25 M potassium phosphate, pH 7.2, in the reservoir. This gives an adequate separation of the three peaks and has the advantage of being much faster than the stepwise procedure. It is advisable to pool the β and γ peaks obtained by this method because of the possibility of cross-contamination. If the column is further eluted with 2.5 M potassium phosphate, pH 7.2, as indicated by the arrow in Fig. 2, a large amount of heme proteins may be eluted. This high salt elution also serves to prepare the column for another fractionation.

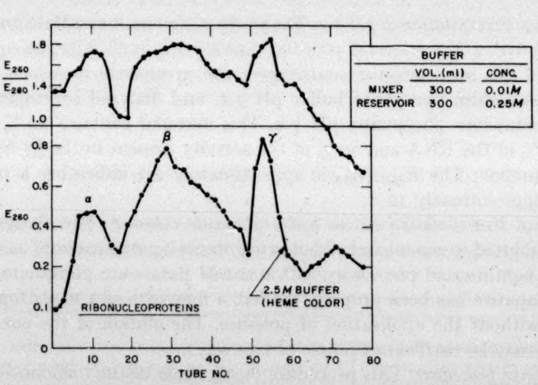

Fig. 2. Linear gradient elution of s-RNA α, β and γ on an hydroxylapatite column.

The three fractions with $E_{260}:E_{280}$ ratios of 1.6 to 1.9 may be pooled separately and lyophilized. The powders obtained are suspended in a small amount of water and dialyzed against 0.08 M potassium phosphate, pH 7.2, or against 0.001 M potassium phosphate, pH 7.2; the 0.08 M phosphate solution is used for assay and the 0.001 M phosphate solution for further fractionation.

Step 5. Fractionation on a DEAE-cellulose column: The fractions dialyzed against 0.001 M phosphate may be separated on a DEAE-cellulose column (N,N-diethylaminoethylcellulose) into a "protein" component and an "RNA" component. Fig. 3 shows the results obtained when 10 ml of the combined fractions of ribonucleoproteins β and γ were added to a 2 cm × 1 cm DEAE column. The elution may be carried out in a stepwise method by the combined use of potassium phosphate buffer, pH 7.2, and sodium chloride solutions of the indicated molarities. The $E_{260}:E_{280}$ ratios of each fraction presented in the same figure indicate that a rather sharp fractionation of the "RNA" from the "protein" component has been achieved. The u.v.-absorbing material that comes through the column in the first 15 ml contains about 50 % of the total protein ($E_{260}:E_{280} = 0.70$ to 0.75) recovered from the column. With increasing salt concentrations several smaller intermediate peaks appear ($E_{260}:E_{280} = 1.0$ to 1.2) and then a large sharp peak appears ($E_{260}:E_{280} = 2.03$ to 2.10) which accounts for over 90 % of the orcinol-reacting material added to the DEAE column. After lyophilization, the material in the peak tubes may be stored in the dry state or the fractions may be assayed after dialysis against 0.08 M potassium phosphate, pH 7.2.

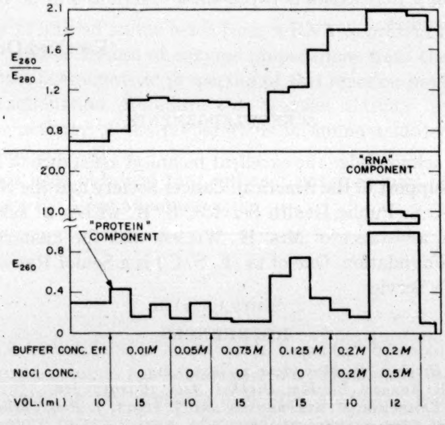

Fig. 3. Stepwise procedure for fractionation of protein from RNA on DEAE column.

General properties

Some of the enzymic properties of the "protein" and of the "RNA" components have already been described. In addition, they appear to contain some pyrophosphatase and phosphatase activity. The ribonucleoprotein fractions α, β and γ obtained from the hydroxylapatite column are free of amino acid-activating enzymes[4], when assayed by the pyrophosphate-ATP exchange technique in the presence and absence of amino acids.

Lyophilized powders of all fractions described are stable for several months at —20°.

NOTE ADDED IN PROOF

s-RNA α, β, and γ are referred to as ribonucleoproteins for the following reasons: (1) the protein and the RNA fractionate together during an extensive purification process, (2) upon continuous gradient elution from the hydroxylapatite columns three well defined and distinct RNA-protein components can be distinguished, (3) when these isolated components are rechromatographed on an hydroxylapatite column each fraction is eluted with the same molarity of salt as before and (4) each component contains its own s-RNA and ribonucleotide incorporating enzyme. The exact type of physical relationship that exists between these fractions will be best elucidated by detailed enzyme studies.

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ACTIVITY OF RAT-LIVER ENZYMES IN THE TRANSFER OF s-RNA-BOUND AMINO ACIDS TO PROTEIN BY RIBONUCLEOPROTEIN PARTICLES

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SUMMARY

1. The transfer of labeled amino acids from s-RNA to protein by purified RNP-particles has been studied by use of enzyme preparations from the soluble fraction of rat liver, and some characteristic properties of this reaction have been described.

2. By a subfractionation procedure this transfer activity has been partially separated from the activity of the preparations in amino acid dependent PP–ATP exchange (amino acid activation).

3. Evidence has been obtained that different enzymes may be involved in the transfer of different s-RNA-bound amino acids.

INTRODUCTION

A transfer of s-RNA-bound, labeled amino acids to protein by incorporation systems containing rat-liver microsomes has been reported by HOAGLAND *et al.*[1]. The reaction was markedly enhanced in the presence of a "pH 5 enzyme fraction". A similar enhancement has more recently been observed by GROSSI AND MOLDAVE[2] with an enzyme preparation from the soluble liver fraction, non-precipitable at pH 5. Since both the pH 5 enzyme fraction and the pH 5 supernatant contain amino acid activating enzymes[3, 4] it may be asked whether or not the activating enzymes are involved also in the transfer reactions[5].

In attempts to elucidate this problem it seemed particularly desirable to employ systems of increased purity. It has previously been shown that purified RNP-particles isolated from rat-liver microsomes can be used with considerable advantage in different kinds of transfer systems[6]. By use of such purified particles the enzymic activities of amino acid transfer and amino acid activation were studied in some detail in subfractions of soluble liver fractions, and characteristic differences in their distribution and general properties were observed.

Abbreviations: RNP-particles, ribonucleoprotein particles; RNA, ribonucleic acid; s-RNA, soluble RNA, precipitable at pH 5; PP–ATP exchange, isotope exchange between [^{32}P]pyrophosphate and ATP; ATP, adenosine triphosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane buffer, pH 7.8; GSH, gluthathione, reduced form; MEA, 2-mercaptoethylamine; TCA, trichloroacetic acid.

EXPERIMENTAL

The livers of Sprague Dawley rats were used. The animals were kept without food for 15–20 h[7] before being killed by decapitation. All preparations were carried out at 0–4°.

Preparation of RNP-particles

RNP-particles were prepared by the method of RENDI AND HULTIN[8] and purified as described previously[6].

Preparation and fractionation of S_{50}-protein

S_{50}-protein was prepared as described by RENDI AND HULTIN[8] (see ref. 9).

The dialyzed solution of S_{50}-protein from 3 rats (approximately 8 mg protein/ml) was treated with 2 volumes of a hydroxylapatite suspension[10], pH 7.2, (150 mg dry weight/ml). A series of fractions was eluted from the apatite with increasing concentrations of potassium phosphate buffer, pH 7.2 (*cf.* Tables V and VI). The suspensions were allowed to equilibrate with the different buffers for at least 20 min, with constant stirring. After each elution the apatite was washed in a larger volume of the same buffer before being treated with the buffer of next higher concentration.

Preparation and fractionation of ammonium sulfate precipitates

Rats with a weight of 100–150 g were used. The livers were minced and homogenized in 2.5 volumes of 0.01 *M* $MgCl_2$ in 0.25 *M* sucrose and were centrifuged in a Spinco preparative ultracentrifuge for 55 min at 105,000 × *g*. The middle part of the supernatant was collected.

Ammonium sulfate (0.32 g/ml) was added to the soluble liver fraction to give 55 % saturation[11]. The suspension was centrifuged after 1 h and the supernatant was brought to 85 % saturation by addition of 0.24 g ammonium sulfate/ml. Both precipitates, Am 0–55 and Am 55–85, were dissolved in 4 ml of 0.2 *M* Tris, centrifuged for 10 min at 12,000 × *g* and dialyzed against 0.002 *M* Tris for 2 h. The fractions were adsorbed on 2 volumes of calcium phosphate gel, pH 6.6 and eluted with potassium phosphate buffers of successively increasing concentrations, as was described for the fractionations of S_{50}-protein (*cf.* Tables II and III).

Preparation of the pH 5 enzyme fraction

The pH 5 enzyme fraction was prepared by the method of HOAGLAND *et al.*[3] with the modification that 0.008–0.01 *M* HCl was used for the precipitation.

Prelabeling of s-RNA with amino acids

s-RNA was prelabeled by the method of HOAGLAND *et al.*[1] with L-[^{14}C]valine, L-[^{14}C]tyrosine or a hydrolysate of ^{14}C-labeled algal proteins. (The hydrolysate had been treated for 10 h at 120° with 10 % HCl and chromatographed twice on Dowex 50 columns[12]. In control experiments no radioactivity was observed in proteins which had been incubated with s-RNA, prelabeled with this purified hydrolysate.)

In order to get a higher content of labeled amino acid in the s-RNA preparations (and higher radioactivities in the final proteins[13]) the pH 5 enzyme fractions in some cases were treated with repeated preincubation–reprecipitation cycles, as described by HECHT *et al.*[14] (preincubated s-RNA).

Measurement of amino acid dependent PP–ATP exchange

The activity of PP–ATP exchange in the different fractions was measured as described previously[15,16]. In many cases an amino acid mixture, containing 3 μmoles of each of 12 amino acids[3] was used. In more detailed studies of the enzyme distribution, however, the amino acids were added separately to the incubation mixture (3 μmoles per tube). The amount of enzyme preparation added is indicated in the tables. Since the activity of amino acid activation was low in most of the enzyme preparations investigated, the incubation time was usually increased to 60 min at 35°. The percentual exchange values were calculated by the equation of DAVIE *et al.*[17].

Measurement of transfer activity

Unless otherwise indicated, the incubation tubes contained in 1.8 ml of 0.025 *M* KCl, 0.01 *M* $MgCl_2$, 0.035 *M* Tris, 0.25 *M* sucrose and 0.01 *M* GSH or MEA: 1 μmole of ATP, 10 μmoles of PEP, 15 μg of pyruvate kinase, 0.2 μmoles of GTP, about 100 μg of prelabeled s-RNA, 0.2 μmoles of the corresponding non-labeled amino acid (or 3.75 μmoles of an amino acid mixture analogous to the amino acid composition of rat-liver proteins[18]), RNP-particles (4–6 mg of protein), and the indicated amount of the protein fractions to be studied. The tubes were incubated for 30 min at 35°. The proteins were precipitated in 5 % TCA, extracted twice with hot TCA, once with warm ethanol, twice with a mixture of ethanol–ether–chloroform (2:2:1), dried with ether and plated on alumina planchets. The radioactivity was measured by a Tracerlab thin mica window counter and the number of total counts per minute was calculated.

Determination of RNA

The content of RNA was determined by the method of CERIOTTI[19], yeast RNA being used as a reference standard.

Determination of protein

The protein content was determined by the method of LOWRY *et al.*[20], crystallized bovine plasma albumin being used as a reference standard.

MATERIALS

ATP, GTP, CTP, Tris, calcium phosphate gel and protamin sulfate were purchased from Sigma Chemical Co. St. Louis, Miss.; PEP and pyruvate kinase from C. R. Boehringer und Soehne, Mannheim; GSH, MEA and non-labeled amino acids from California Foundation, Los Angeles, Cal.; L-[^{14}C]valine from Institut Pasteur, Paris and the Radiochemical Centre, Amersham (74mC/mmole, and 8 mC/mmole, respectively); L-[^{14}C]tyrosine (11.6 mC/mmole), ^{14}C-algal-protein hydrolysate (0.088 mC/mg) and [^{32}P]phosphate from the Radiochemical Centre, Amersham; yeast RNA from Hopkins and Williams Ltd., Ilford; bovine plasma albumin from Armour Lab., Chicago Ill.; puromycin from Lederle Laboratory Division, New York; chloramphenicol from Parke Davis, Hounslow. Hydroxylapatite was prepared by the method of TISELIUS *et al.*[10], PEP was recrystallized according to BAER[21].

RESULTS

Participation of soluble liver fractions in the transfer of s-RNA-bound amino acids to protein by RNP-particles

The enhancing effects of soluble liver fractions on the transfer of s-RNA-bound amino acids to protein in systems containing purified RNP-particles was studied in a series of introductory experiments. The s-RNA preparations used in these experiments had been prelabeled with L-[^{14}C]valine or a mixture of L-[^{14}C]amino acids. They were incubated with purified particles in the presence of a nucleoside triphosphate generating system and GTP or with only GTP (5 μmoles)[22]. Since there possibly might be some release of labeled amino acid from s-RNA under these conditions, the corresponding L-[^{12}C]amino acids were included in the systems in at least 100-fold excess to prevent any incorporation of isotope by way of the free amino acid pool.

In the absence of soluble liver enzymes only a limited isotope incorporation was observed and the addition of the SH-compounds MEA or GSH had no appreciable effect (Fig. 1).

When small amounts of a pH 5 enzyme fraction were included in the incubation systems, the labeled amino acids were readily transferred from s-RNA and incorporated into protein. Enzyme preparations, which had been stored for some time at —20° were significantly stimulated by SH-compounds. A similar stimulation was observed also with freshly prepared enzyme, especially at higher concentrations.

A rapid isotope incorporation was obtained also when S_{50}-protein was added to

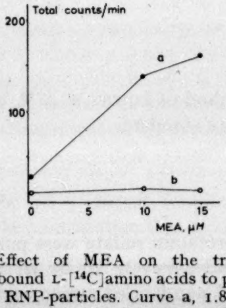

Fig. 1. Effect of MEA on the transfer of s-RNA-bound L-[^{14}C]amino acids to protein by purified RNP-particles. Curve a, 1.8 mg S_{50}-protein present; curve b without soluble liver enzymes. The incubation tubes contained in 1.8 ml of 0.025 *M* KCl, 0.01 *M* $MgCl_2$, 0.035 *M* Tris and 0.25 *M* sucrose: 1 μmole of ATP, 10 μmoles of PEP, 15 μg of pyruvate kinase, 0.2 μmole of GTP, 3.75 μmoles of an amino acid mixture, RNP-particles (5 mg of protein), 50 μg of prelabeled s-RNA (1600 counts/min) and the amount of MEA indicated. Incubation for 30 min at 35°.

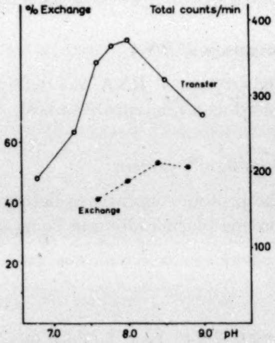

Fig. 2. Effect of pH on transfer activity and amino acid dependent PP–ATP exchange activity of liver enzyme preparations. Transfer experiment: Incubation system as in Fig. 1, but with 10^{-2} *M* GSH, 6 mg S_{50}-protein, RNP-particles corresponding to 4 mg of protein and Tris buffer at different pH. PP–ATP exchange experiment: The tubes contained in a final volume of 1.3 ml: 4 μmoles of [^{32}P]pyrophosphate, 6 μmoles of ATP, 6 μmoles of $MgCl_2$, 60 μmoles of KF, 3 μmoles of each of 12 amino acids[3], 50 μmoles of Tris buffer at different pH and 0.7 mg of pH 5 enzyme protein. Incubation for 12 min at 35°.

the incubation system in combination with MEA or GSH. The stimulation by the SH-compounds was very marked in experiments of this kind (Fig. 1).

Characteristic properties of transfer systems containing S_{50}-protein

On the basis of equal protein content the activity of the pH 5 enzyme fraction in the transfer systems was considerably higher than that of the 50-S-protein[6]. In spite of this fact a particular attention was paid to the properties of the transfer systems with S_{50}-protein. This preparation had a relatively low activity of amino acid activating enzymes (except for tyrosine, proline and cysteine activating enzymes) and no soluble RNA could be observed even by use of radioactivity measurements after pre-labeling with large doses of [^{32}P]phosphate *in vivo*[6].

The effect of pH on the rate of amino acid transfer by a system with S_{50}-protein is illustrated by Fig. 2. A fairly sharp activity optimum was found near pH 8. For comparison, the pH curve for amino acid dependent PP–ATP exchange by a pH 5 fraction, incubated with a mixture of 12 amino acids[3], is included in the figure. The optimum activity of this system was at about pH 8.4 with only a slight decrease on either side.

Under optimum conditions 30–40% of the s-RNA-bound L-[^{14}C]amino acids were transferred to protein by the system. With S_{50}-protein added in some excess this transfer was completed in about 5 min. Approximately the same incorporation level was reached, however, with lower concentrations of S_{50}-protein, if the incubation periods were increased to 30 min or more. A preincubation of the particles for 20 min (35°) before the additon of L-[^{14}C]valine-s-RNA, S_{50}-protein, GTP and the nucleoside triphosphate generating system did not significantly decrease the rate of L-[^{14}C]valine-transfer.

As is shown by Fig. 3 the amount of L-[^{14}C]valine transferred by the system was a linear function of the amount of L-[^{14}C]valine-s-RNA added. Within certain limits, the reaction proceeded at a rate proportional to the amount of S_{50}-protein added to the system (Fig. 4).

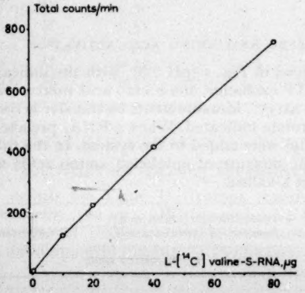

Fig. 3. Dependence of transfer rate on substrate concentration. Incubation system as in Fig. 1, but with 10^{-2} *M* GSH, 0.2 µmole of L-valine instead of the amino mixture, 4 mg of S_{50}-protein and 5.7 mg of RNP-particle protein and the indicated amounts of preincubated (*cf.* EXPERIMENTAL) L-[^{14}C]valine-s-RNA (10 [illegible] corresponded to 250 counts/min).

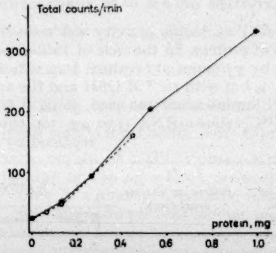

Fig. 4. Dependence of transfer rate on enzyme concentrations. ●—●, whole S_{50}-protein; ○—○, 0.15 *M* subfraction of S_{50}-protein. Incubation system as in Fig. 3, but with RNP-particles, corresponding to 6.5 mg of protein, 30 µg of preincubated L-[^{14}C]valine-s-RNA (750 counts/min), and the indicated amounts of enzyme.

The transfer reaction was inhibited by puromycin[23] and to a minor extent by chloramphenicol (Table I).

Amino acid transfer and amino acid activation by subfractions of soluble liver fractions

On the basis of the information obtained about the transfer systems the possible relationship between amino acid transfer and amino acid activation was further studied in a series of subfractionation experiments. Experiments of this kind were carried out with the ammonium sulfate fractions Am 0–55 and Am 55–85 of the whole soluble liver fraction, and with S_{50}-protein preparations.

The ammonium sulfate fractions Am 0–55 and Am 55–85 were adsorbed on calcium phosphate gel and eluted with potassium phosphate buffers (pH 7.2) of stepwise increasing concentrations (0–0.6 *M*). The activities of amino acid transfer and amino acid dependent PP–ATP exchange observed in the different subfractions are shown in Tables II and III.

With a mixture of 12 amino acids[3], PP–ATP exchange activity was found in both ammonium sulfate fractions, although with very different patterns of distribution.

TABLE I

EFFECTS OF CHLORAMPHENICOL AND PUROMYCIN ON THE TRANSFER OF LABELED AMINO ACIDS FROM s-RNA TO PROTEIN

Incubation system as in Fig. 1, but with 10^{-2} *M* GSH and 4 mg S_{50}-protein. The prelabeled s-RNA (100 μg, 1000 counts/min) was added after a preincubation of 10 min at 23°. Further incubation 30 min at 35°.

	Total counts/min
Complete system	117
Complete system, plus 10^{-3} *M* chloramphenicol	124
Complete system, plus $5 \cdot 10^{-3}$ *M* chloramphenicol	103
Complete system, plus $2 \cdot 10^{-6}$ *M* puromycin	100
Complete system, plus $2 \cdot 10^{-3}$ *M* puromycin	18

TABLE II

ACTIVITIES OF Am 0–55 SUBFRACTIONS IN TRANSFER AND AMINO ACID ACTIVATION

The PP–ATP exchange activity was measured as described in Fig. 2 (pH 7.8), with the indicated amounts of protein. In the case of valine specific PP–ATP exchange, the amino acid mixture was replaced by 3 μmoles of L-valine. Incubation for 60 min at 35°. Measurements of transfer activity as in Fig. 1, but with 10^{-2} *M* GSH and the amounts of protein indicated. When s-RNA, prelabeled with L[^{14}C]amino acids was used, 50 μg (1,600 counts/min) were added to the system. In the tubes with L-[^{14}C]valine-s-RNA (150 μg, 300 counts/min), the mixture of unlabeled amino acids was replaced by 0.2 μmole of L-valine.

Buffer for elution	*Protein in enzyme assay (mg)*	*% exchange*		*Isotope transfer (counts/min) from s-RNA, prelabeled with*		*RNA/protein* × 10^{-3}
		Valine	*Amino acid mixture*	L-[^{14}C]*valine*	L-[^{14}C]*amino acids*	
0	1.66	0	0	3	23	<0.7
0.001 *M*	0.09	0	0.6	1	6	0
0.05 *M*	1.39	0	10.2	1	14	1.6
0.1 *M*	0.49	0	4.7	1	13	<0.7
0.2 *M*	0.50	0.2	17.0	72	156	—
0.3 *M*	0.36	17.0	48.0	51	128	1.2
0.6 *M*	0.22	5.3	18.0	5	20	<0.7

With valine alone, exchange activity was observed only in Am 0–55, and more exactly in two consecutive subfractions eluted with 0.3 and 0.6 *M* phosphate.

The transfer activity, as measured both with a mixture of s-RNA-bound L-[^{14}C]-amino acids and with L-[^{14}C]valine-s-RNA showed a distribution which was characteristically different from that of the PP–ATP exchange activities. As might be anticipated, the difference was most distinct in experiments involving only one amino acid, *e.g.* valine. The subfraction of the Am 0–55 fraction eluted with 0.2 *M* phosphate was of a particular interest, since the L-[^{14}C]valine transfer by this subfraction was relatively high, while the valine dependent PP–ATP exchange was almost insignificant. A more detailed study of this subfraction was therefore undertaken (Table IV). Not only with L-[^{14}C]valine-s-RNA but also with L-[^{14}C]tyrosine-s-RNA a relatively high transfer was obtained, in spite of the very low PP–ATP exchange activities with these amino acids.

Subfractionation experiments of a similar kind were carried out with the S$_{50}$-protein preparations. The fractionations, however, in this case were made with hydroxylapatite[10] instead of calcium phosphate gel. An experiment of this kind is shown in Table V. With the mixture of 12 amino acids the PP–ATP exchange activities were low and concentrated in the 0.3 *M* and 0.6 *M* subfractions. On the other hand, the transfer activity, as determined with the mixture of s-RNA-bound [^{14}C]amino acids was highest in the 0.2 *M* subfraction. A definite transfer was obtained already

TABLE III

ACTIVITIES OF AM 55–85 SUBFRACTIONS IN TRANSFER AND AMINO ACID ACTIVATION

For details see Table II.

Buffer for elution	*Protein in enzyme assay (mg)*	*% exchange*		*Isotope transfer (counts/min) from s-RNA, prelabeled with*		*RNA/protein × 10^{-3}*
		valine	*amino acid mixture*	L-[^{14}C]*valine*	L-[^{14}C]*amino acids*	
0	2.43	0	25.2	214	294	2.3
0.001 *M*	0.18	0.5	2.6	5	30	18.0
0.05 *M*	0.30	0	0	0.4	31	4.3
0.1 *M*	0.31	0	6.0	2	9	19.4
0.2 *M*	0.44	0.5	11.5	10	26	7.3
0.3 *M*	0.17	0.2	43.8	14	36	0
0.6 *M*	0.11	0	2.7	1	18	0

TABLE IV

ACTIVITIES OF AM 0–55 SUBFRACTIONS IN TRANSFER AND AMINO ACID ACTIVATION

For details see Table II. Isotope transfer system, with 150 μg of L-[^{14}C]valine-s-RNA (500 counts/min), 180 μg L-[^{14}C]tyrosine-s-RNA (200 counts/min), or 100 μg s-RNA prelabeled with a mixture of [^{14}C]amino acids (1000 counts/min). L-valine (0.2 μmole), L-tyrosine (0.2 μmole) and an amino acid mixture (3.75 μmoles), respectively, were added to prevent isotope incorporation from free amino acids.

Buffer for elution	*Protein in enzyme assay (mg)*	*% exchange*			*Isotope transfer (counts/min) from s-RNA, prelabeled with*		L-[^{14}C]*amino acids*
		Valine	*Tyrosine*	*Amino acid mixture*	L-[^{14}C]*valine*	L-[^{14}C]*tyrosine*	
0.15 *M*	1.18	0.9	0.6	5.6	5	2	11
0.19 *M*	0.76	1.5	0	10.3	54	23	85
0.23 *M*	0.52	1.4	1.2	13.2	67	19	107

with the 0.1 *M* subfraction by which only an insignificant amino acid dependent PP–ATP exchange was catalyzed.

The activity of valine dependent PP–ATP exchange was concentrated in the 0.4 *M* subfraction, although some activity was also found in the 0.2 *M* fraction (Table VI). The same was true of the tyrosine dependent exchange. In the 0.4 *M* subfraction this latter exchange activity was relatively high (it should be noted that the exchange experiments with the mixture of 12 amino acids[3] did not include tyrosine).

The transfer ratio of [^{14}C]valine to [^{14}C]tyrosine was not constant in the different subfractions (Table VI). The activity of [^{14}C]tyrosine transfer was highest in the 0.2 *M* fraction, while the maximum activity of [^{14}C]valine transfer was not reached until later. As in the case of the [^{14}C]amino acid mixture (Table V) an activity of [^{14}C]-tyrosine transfer was observed already in the 0.1 *M* fraction, in which there was no evidence of valine or tyrosine activating enzymes.

The PP–ATP exchange ratios of valine to tyrosine were different from the corresponding transfer ratios. In the case of the 0.4 *M* subfraction the tyrosine activation was about 10 times higher than the valine activation, while, on the other hand, the transfer of tyrosine was considerably less active than the transfer of valine (Table VI).

TABLE V

ACTIVITIES OF S_{50}-PROTEIN SUBFRACTIONS IN TRANSFER AND AMINO ACID ACTIVATION

See Table II for details.

Buffer for elution	*Protein in enzyme assay (mg)*	*% exchange amino acid mixture*	*Isotope transfer (counts/min) from s-RNA, prelabeled with [^{14}C]amino acids*
0	1.32	0	124
0.001 *M*	0.40	0	13
0.05 *M*	1.0	0.43	11
0.1 *M*	1.70	0.45	54
0.2 *M*	1.08	2.2	248
0.3 *M*	1.28	7.40	179
0.6 *M*	0.40	3.3	28

TABLE VI

TRANSFER AND ACTIVATION OF VALINE AND TYROSINE BY SUBFRACTIONS OF S_{50}-PROTEIN

See Table II for details. Measurement of transfer activity as in Table II, but with 70 μg (400 counts/min) of L-[^{14}C]valine-s-RNA or 150 μg of L-[^{14}C]tyrosine-s-RNA (220 counts/min), 0.2 μmole of L-valine or L-tyrosine instead of the amino acid mixture and the amounts of protein indicated.

Buffer for elution	*Protein in enzyme assay (mg)*	*% exchange*		*Isotope transfer (counts/min) from s-RNA, prelabeled with*	
		Valine	*Tyrosine*	L-[^{14}C]*valine*	L-[^{14}C]*tyrosine*
0.05 *M*	1.0	1.4	1.3	15	9
0.1 *M*	1.0	0.3	0	41	13
0.2 *M*	1.0	0.3	0.3	65	26
0.4 *M*	0.5	5.5	53.4	113	13
Original S_{50}-protein	1.0	0.4	14.4	143	27

Like the 0.1 *M* fraction the eluate with 0.15 *M* phosphate had no measurable activity of amino acid dependent pyrophosphate exchange. Some properties of this fraction were studied for comparison with the S_{50}-protein. The transfer activity of the 0.15 *M* fraction was stimulated by GSH (10^{-2} *M*). The enzyme concentration curve is included in Fig. 4.

DISCUSSION

The experiments reported here suggest that the amino acid activation and the transfer of s-RNA-bound amino acids to RNP-particles are catalyzed by different enzymes, and the following evidence may be presented in favor of this view. (a) The transfer activity was stimulated by MEA and GSH, while the activation of amino acids, as measured by amino acid dependent PP–ATP exchange, does not require the SH-compounds[24]. (b) The activation and transfer reactions have slightly different pH optima. The possibility should be kept in mind, however, that the lower pH optimum of the transfer reaction may be related to an increased instability of amino acid-s-RNA at higher pH (see ref.[25]). (c) By fractionation of liver enzyme preparations on calcium phosphate gel or hydroxylapatite, transfer activity was obtained in subfractions, which did not show any corresponding amino acid dependent PP–ATP exchange activity. (d) In overlapping subfractions there was no direct correlation between the activities of amino acid activation and amino acid transfer. (e) The S_{50}-protein had a relatively high content of tyrosine activating enzyme, which mainly appeared in the 0.4 *M* subfraction (Table VI). The isotope transfer from L-[^{14}C]tyrosine-s-RNA was rather low, however, and less transfer activity was shown by this fraction than by that eluted with 0.2 *M* phosphate.

The different ratios of valine to tyrosine transfer in the individual subfractions of S_{50}-protein (Table VI) may possibly indicate that different enzymes are involved in the transfer of different amino acids.

The non-amino acid dependent PP–ATP exchange is little understood at present. As a rule, this activity was relatively low in the S_{50}-protein. A high activity, however, was shown by some subfractions of the ammonium sulfate precipitates (particularly in Am 55–85). No correlation was observed between this non-amino acid dependent exchange and the RNA content. As a matter of fact, by this subfractionation method RNA was largely separated also from the amino acid dependent exchange activities[26].

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A HYDROLYTIC PROCEDURE FOR RIBONUCLEOSIDES AND ITS POSSIBLE APPLICATION TO THE SEQUENTIAL DEGRADATION OF RNA

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SUMMARY

Ribonucleosides are converted quantitatively to the corresponding bases by reacting successively with periodate and cyclohexylamine. The hydrolysis of the glycosidic linkage of the nucleoside is presumably due to the formation of a double Schiff base between the periodate oxidized nucleoside and cyclohexylamine. This method has been also applied to the hydrolysis of the terminal nucleoside of s-RNA. The molar ratios of the free bases (uracil:adenine:cytosine, 1:2.8:6.1) thus obtained from the terminal nucleosides of RNA were found to be the same as those of the terminal nucleosides (uridine:adenosine:cytidine, 1:2.9:6.1) obtained by alkaline hydrolysis of RNA of the same preparation. The phosphoester linkage between the diesterphosphate and the 5′-hydroxyl group of the oxidized terminal nucleoside of RNA was also found to be hydrolyzed during the cyclohexylamine treatment, thus exposing a new terminal 3′-phosphate. It has been shown that, following prostatic phosphomonoesterase treatment, the periodate and cyclohexylamine reactions can be repeated and thus a possible method for the sequential degradation of RNA is provided. An application of this method to the sequential degradation of phenol extracted s-RNA and preliminary results thereof have been discussed.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

INTRODUCTION

Most procedures for the hydrolysis of ribonucleosides involve the use of either strong acid or strong alkali at rather high temperatures[1]. WHITFELD[2] has reported a milder procedure in which the ribonucleoside is first oxidized with periodate. Free base is obtained after incubation of the periodate oxidation products with glycine buffer at pH 10 for 18 h at 37°. This procedure has also been applied to the degradation of di- and tri-nucleotides. Uracil and 5-bromouracil have been obtained when periodate oxidized uridine (or 5-ribosyluracil) is treated with bromine[3].

The present communication reports a hydrolytic procedure for ribonucleosides in which the periodate oxidation products of ribonucleosides are treated with cyclohexylamine. Quantitative conversions of the nucleosides to the corresponding bases are obtained. This procedure has also been applied to the hydrolysis of the terminal nucleosides of RNA. Use of cyclohexylamine for this purpose has been suggested by HAKOMORI[4]. The phosphoester linkage between the diester phosphate and the oxidized terminal nucleoside is also hydrolyzed under these conditions, thus exposing a new terminal 3′-phosphate. Successive treatments with phosphomonoesterase and a repetition of the periodate and cyclohexylamine procedure may provide a method for the sequential degradation of RNA. Preliminary results in the application of this procedure to the sequential degradation of the phenol extracted RNA from yeast[5] will be discussed.

EXPERIMENTAL AND RESULTS

Material and methods

The nucleosides were purchased from the California Corporation for Biochemical Research, Los Angeles, California. s-RNA from yeast was prepared according to the method of MONIER *et al.*[5] Prostatic phosphomonoesterase was kindly supplied by Dr. G. SCHMIDT.

The paper electrophoretic experiments were carried out according to the procedure of CRESTFIELD AND ALLEN[6]. Whatman No. 1 paper which was washed with hot 2 *N* acetic acid and rinsed with distilled water was used throughout the experiments[7].

Periodate oxidation of ribonucleosides

To 0.5 ml of a 0.2 *M* solution of uridine (100 µmoles), 1 ml of a 0.2 *M** solution of potassium periodate (200 µmoles) was added. In contrast with reports from many laboratories[2,8], the periodate oxidation was found to be completed immediately after the reagents were added together. Similar experiments using adenosine and cytidine were carried out. Because of the low solubility of guanosine in aqueous solutions, a more dilute solution (0.01 *M*) was used.

Reactions of cyclohexylamine with periodate oxidized ribonucleosides

Various amounts of cyclohexylamine were added to individual samples of the periodate oxidized ribonucleosides. The final concentrations of the cyclohexylamine in the reaction mixture were 0, 0.06, 0.12, 0.24, 0.48, 0.96 and 1.92 *M*. (The molar ratios of the cyclohexylamine and the oxidized ribonucleosides used in these experiments were 0, 0.9, 1.8, 3.6, 7.2, 14.4 and 28.8.) Samples of the reaction mixture were spotted for paper chromatography immediately after the addition of cyclohexylamine.

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* See NOTE ADDED IN PROOF.

The chromatograms were developed with either isobutyric acid–0.5 *M* ammonium hydroxide (10:6, v/v) (see ref.[9]) or isopropanol–acetic acid–water (60:30:10, v/v/v) (see ref.[10]) solvents. As shown in Fig. 1A, the quantity of the free base formed increases with the increasing concentration of cyclohexylamine and is substantially quantitative at 1.92 *M* cyclohexylamine (No. 7, Fig. 1A). The amounts of the free bases recovered range from 93 to 100% of the four ribonucleosides used in the experiments.

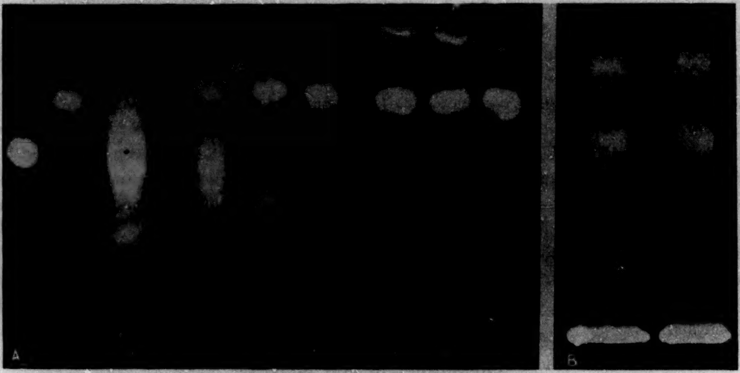

Fig. 1. A. Uridine treated with periodate and cyclohexylamine. First and second column, from left, reference compounds uridine and uracil respectively. Number 1 to 7, aliquots of periodate oxidized uridine to which cyclohexylamine was added to a final concentration of 0, 0.06, 0.12, 0.24, 0.48, 0.96 and 1.92 *M* respectively. Small quantities of U.V.-absorbing material behind uracil which have not been identified are presumably the oxidized ribose and its reaction product with cyclohexylamine. Paper chromatograms were developed with isobutyric acid–0.5 *N* ammonium hydroxide solvent ascending for 12 h. B. RNA treated with periodate and cyclohexylamine. The heavy white spot at the origin is the bulk of the RNA molecules. The three U.V.-absorbing spots are, from the top, uracil, adenine and cytosine respectively. The paper was developed in butanol–water solvent ascending for a total of six 12-h periods.

Hydrolysis of terminal nucleosides of s-RNA with periodate and cyclohexylamine

Samples of 20 mg of s-RNA were dissolved in 0.2 ml water and were oxidized with 0.2 ml periodate (0.2 *N*). It is known that only the terminal free nucleosides of RNA are oxidized by periodate[11]. Cyclohexylamine was added to a final concentration of 1 *M*. Some of the samples were spotted for ascending paper chromatography and others were dialyzed overnight at 4°. The papers were developed with either butanol–water (84:16, v/v) (see ref.[8]) or isopropanol–acetic acid–water solvents. When the butanol–water solvent was used, the paper was dried after a 12-h development and resubmitted to the same solvent. This process was repeated several times in order to separate the free bases from the bulk of the RNA molecules. As shown in Fig. 1B, the bulk of the RNA molecules remained at the site of application. The u.v.-absorbing material with the highest R_F value was identified as uracil, that with the second R_F value as adenine, and that with the lowest R_F value as cytosine. The identification of these compounds was made by comparison of their R_F values, electrophoretic mobilities at pH 3.5 and u.v.-absorbing spectra at pH 1 and pH 13 with those of known reference compounds. The molar ratios of the free bases obtained are 1:2.8:6.1

(uracil: adenine: cytosine). When the isopropanol–acetic acid–water solvent was used, a single 12-h development was sufficient to achieve the separation of the free bases from the bulk of the RNA molecules. Since adenine and cytosine have similar R_F values in this solvent, an additional chromatographic procedure with butanol-water solvent system was required to separate these two compounds.

To each of the dialyzed samples of periodate oxidized, cyclohexylamine treated s-RNA, 10 units* of prostatic phosphomonoesterase was added, and the mixtures were incubated for 15 min at 37° in 0.1 *N* acetic acid buffer at pH 5.6. Thereafter, the samples were mixed thoroughly with equal volumes of 90 % phenol and centrifuged for 15 min at 3,000 rev./min at room temperature. The upper layer was recovered and to it were added 10 % by volume of 20 % potassium acetate at pH 5 and 2 volumes of 95 % cold ethanol. The samples were left in the deep freeze for at least 4 h. The RNA precipitates were recovered by centrifugation, dissolved in small amounts of water, and dialyzed overnight. After dialysis the RNA samples were treated with periodate and cyclohexylamine and were subjected to paper chromatography as described. The free bases obtained were presumably thus derived from the nucleotides next to the original terminal nucleosides of the s-RNA molecules. From these second nucleotides of the RNA molecules only adenine and cytosine were found, and the molar ratio of adenine to cytosine was 1:3.9. Control experiments were carried out in which either the periodate or the cyclohexylamine or the phosphomonoesterase treatments were omitted. No detectable base was found during the subsequent paper chromatography in any of the control experiments. These preliminary observations indicate that this procedure is promising in the sequence determination of RNA. Experiments are under way to carry the degradation further into the RNA molecules.

Alkaline hydrolysis of s-RNA

In order to verify the results on the terminal groups of RNA obtained by the periodate and cyclohexylamine treatments, a sample of RNA from the same preparation was hydrolyzed with alkali and the terminal nucleosides liberated were determined. 60 mg of s-RNA were hydrolyzed with 0.8 ml of 1 *N* KOH at room temperature for 24 h. Thereafter the solution was neutralized with concentrated perchloric acid and centrifuged at 3,000 rev./min for 10 min at 2°. The precipitate was washed with 0.5 ml of water and the suspension was again centrifuged in the cold. The combined supernatant solutions were spotted for paper chromatography in butanol–water solvent ascending for 12 h. The papers were then dried and resubmitted to the same solvent for a total of six 12-h periods. The nucleosides moved from the origin in this solvent system, whereas the nucleotides remained at the site of application. The area of the paper containing the nucleosides was cut horizontally into four approx. ½″ wide strips and eluted with water. The material eluted from the paper was resubmitted to paper chromatography with the isobutyric acid–0.5 *N* ammonium hydroxide solvent ascending for 12 h. The nucleosides thus completely separated from each other were identified as uridine, adenosine and cytidine by comparison of their R_F values and their u.v.-absorbing spectra at pH 1 and pH 13 with those of known reference compounds. A total of 1.94 µmoles of the nucleosides were obtained and the molar ratios of uridine, adenosine and cytidine were found to be 1:2.9:6.1. These nucleosides were presumably derived from the terminal nucleosides of RNA molecules[12]. The molar ratios of the nucleosides were approximately the same as those of the bases derived from the terminal nucleosides of RNA by the periodate and cyclohexylamine method. Assuming that no branching of the s-RNA chain occurs, these data are consistent with a molecular weight of around 30,000.

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* Each unit of prostatic phosphomonoesterase equals 10 µl of an enzyme preparation which is capable of hydrolyzing 1 µmole of adenosine-3′-phosphate in 15 min at 37° in 0.1 *N* acetic acid buffer at pH 5.6. No diesterase activity was detectable under these conditions.

DISCUSSION

The reactions which lead to the formation of free base from the periodate oxidation product of ribonucleoside probably involve the formation of an intermediate which is a double Schiff base between the oxidized ribonucleoside and cyclohexylamine (the compound in parentheses, Fig. 2). BARRY AND MITCHELL[13] reported that crystalline Schiff base was formed when the periodate oxidized starch was treated with cyclohexylamine. The Schiff base formed was found to consist of two moles of cyclohexylamine/mole of the oxidized glucose unit of starch. GUTHRIE *et al.*[14] reported the formation of a similar compound when periodate oxidized methyl 4,6-O-benzylidene-2-D-glucoside was treated with cyclohexylamine. The hydrolysis of the glycosidic linkage then can be attributed to the nucleophilic property of the nitrogen atom of cyclohexylamine, which may result in a redistribution of electrons along the chain of the oxidized ribose molecule. The probable reactions leading to the formation of free base from ribonucleoside are described in Fig. 2.

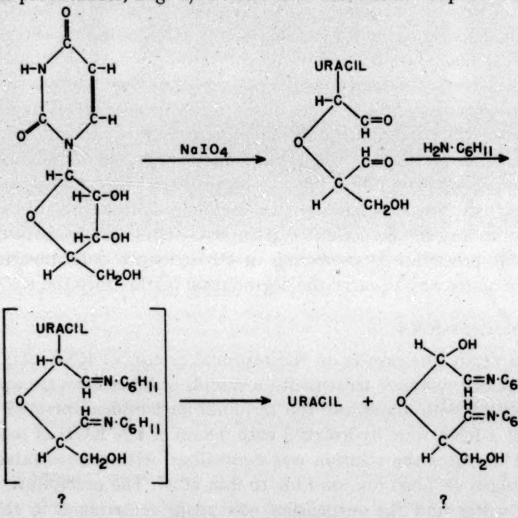

Fig. 2. Proposed reactions leading to the formation of free bases from the corresponding ribonucleosides when treated successively with periodate and cyclohexylamine.

Similar reactions with periodate and cyclohexylamine are conceivable for the hydrolysis of the glycosidic linkage of the terminal nucleoside groups of RNA (see Fig. 3). The hydrolysis of the phosphoester linkage between the diester phosphate and the oxidized terminal nucleoside can also be attributed to the redistribution of electrons resulting from the formation of the Schiff base. The hydrolysis of this phosphoester linkage would result in the formation of a new terminal 3′-phosphate. In agreement with this postulation, Burton and Peterson[15] report that after removing the purines from DNA, the phosphoester linkages of the original purine nucleotides in DNA become labile in the presence of diphenylamine. The formation of a compound between the aldehyde group of apurinic acid and diphenylamine was suggested as a possible mechanism for the hydrolysis of the phosphoester linkage.

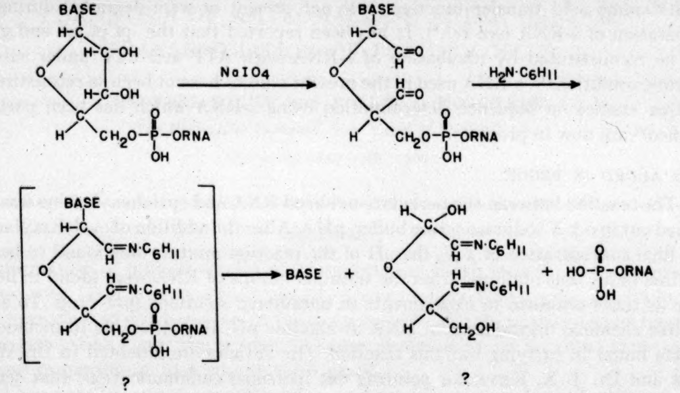

Fig. 3. Proposed reactions leading to the formation of free bases from the terminal nucleosides of RNA when treated successively with periodate and cyclohexylamine.

After removal of the terminal phosphate with phosphomonoesterase, a new terminal nucleoside is exposed and the above procedure of periodate and cyclohexylamine reactions can be repeated, and thus a potential procedure is provided for the sequential degradation of RNA. In Whitfeld's[2] procedure for the degradation of di- and tri-nucleotide, the periodate oxidized polynucleotide is incubated with buffer at pH 10 at 37° for 18 h. In the present studies the reactions are carried out at room temperature and are almost instantaneous, thus minimizing the danger of other chemical or enzymic degradation of RNA during the experiments. The terminal nucleosides of RNA determined with this procedure have been compared with those obtained from alkaline hydrolysis of RNA of the same preparation, and have been found to consist of similar proportions of uridine, adenosine and cytidine.

The phenol extracted RNA from yeast used in the present studies was found to be comparable with the soluble RNA (s-RNA) in its physical, chemical and biochemical properties[5]. s-RNA participates in protein synthesis by forming an amino acyl-RNA compound in which the amino acid is attached to the 3′ (or 2′) hydroxyl group of the terminal nucleoside adenosine[16–19]. s-RNA in its amino acid acceptor form presumably has a terminal sequence of -pCpCpA (see ref.[19]). Singer and Cantoni[20] have reported that the terminal nucleoside of s-RNA as extracted from rat liver consists of adenosine, cytidine and small amounts of other nucleosides, and studies the terminal nucleosides of s-RNA extracted from yeast were found to consist of uridine (10 %), adenosine (29 %) and cytidine (61 %). The discrepancies between that adenosine represents at least 50 % of the total terminal nucleosides. In the present these results and those which indicate the terminal -pCpCpA structure for s-RNA may be explained by assuming that some of the terminal groups of s-RNA necessary for its amino acid transfer function were not present or were degraded during the preparation of s-RNA (see ref.[5]). It has been reported that the -pCpCpA end group can be reconstituted by incubation of s-RNA with ATP and CTP under suitable enzymic conditions[16]. s-RNA used in the present studies has not been so reconstituted. Further studies on sequence determination using s-RNA which has been partially purified[21] are now in progress.

NOTE ADDED IN PROOF

The reaction between the periodate oxidized RNA and cyclohexylamine was also carried out in 0.7 *N* sodium acetate buffer, pH 5. After the addition of cyclohexylamine to a final concentration of 1 *N*, the pH of the reaction mixture was found to be 6.2. The free bases thus obtained from the terminal groups of RNA were found to be the same as those obtained in experiments in unbuffered solutions (pH 11.9). To avoid possible chemical degradation of RNA at alkaline pH, it is desirable to include the acetate buffer in carrying out this reaction. The authors are indebted to Dr. W. E. COHN and Dr. J. X. KHYM for pointing out (personal communication) that certain amine induced cleavages of periodate oxidized nucleotide residues are favored at acid pH's.

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FORMATION OF AMYLOMALTASE AFTER GENETIC TRANSFORMATION OF PNEUMOCOCCUS

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SUMMARY

Transformation of mutant strains of pneumococcus which lack the enzyme amylomaltase with DNA from a wild strain which can produce the enzyme, results in the rapid formation of amylomaltase in the recipient cells. Within 10 min, a small fraction of the generation time, the enzyme is being produced at its maximal rate—as a constant proportion of new protein synthesized.

When parallel cultures of different mutant strains are transformed, although the cells are believed to take up the same amounts of DNA, the rate of formation of amylomaltase is proportional to the extent of permanent genetic change, which is different for the different recipient populations. This suggests that DNA must not only be incorporated but must be permanently integrated in the cell in order to elicit enzyme synthesis.

Transformation of a mutant strain with DNA from another strain mutated at a different but linked site, also results in the rapid formation of amylomaltase. In this case enzyme formation requires that donor DNA interact with host DNA, probably by some form of genetic recombination.

Thus, in the process of transformation, it appears that integration, recombination, and probably replication of newly-introduced DNA, as well as its elicitation of specific protein synthesis, occur very soon after entry of the DNA into the cell.

INTRODUCTION

The genetic transformation of pneumococcus by deoxyribonucleate[1] affords an opportunity to study the manner in which the genetic material effects the synthesis of specific proteins. It has been previously reported that mutant strains of pneumococcus lacking an enzyme essential for the utilization of maltose, when transformed with DNA give rise to maltose-utilizing cells[2]. In the present investigation the appearance of the enzyme in transformed cultures has been followed.

DNA from a maltose-utilizing wild strain is able to transform different mutant strains to maltose-utilization, but for each strain this transformation occurs with a characteristic frequency. Furthermore, in certain cases DNA from one mutant strain

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Abbreviations: DNA, deoxyribonucleate; Tris, tris(hydroxymethyl)aminomethane.

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can transform cells from another to maltose-utilization, and a genetic analysis based on the frequencies of such recombination indicated that the different mutations represent alterations in different, closely-linked regions of the genetic material[2].

The enzyme which is present in the wild strain but absent in the mutant strains is a transglycosidase which cleaves maltose to give glucose and oligosaccharides[2]. Since its action on maltose appears to be similar to that of the enzyme of *E. coli* called amylomaltase[3], it will also henceforth be referred to as amylomaltase.

EXPERIMENTAL TECHNIQUES

The manner in which the amylomaltase-deficient mutant strains of pneumococcus, Mc, Md, Me, Mf, Mg, and Mi, were obtained has been previously described[2]. DNA preparations were made from R6S, a non-encapsulated derivative of a wild strain which contained, also, a marker for streptomycin-resistance, and from MfS, a mutant strain into which the streptomycin-resistance marker had been incorporated by means of transformation. DNA was prepared essentially according to the method of McCarty and Avery[4].

Cultures of the mutant strains were grown for transformation in 400-ml batches of a casein hydrolysate–vitamins–salts medium supplemented with bovine serum albumin, yeast extract, and glucose as previously described[2]. When the turbidity corresponded to about $4 \cdot 10^7$ colony-forming units/ml, 40 ml of glycerol were added, and the culture was frozen. Within several days, the culture was thawed, centrifuged, and the cells were resuspended in 40 ml of medium. Transformation was accomplished according to the method of Fox and Hotchkiss[5]. DNA was added to give a concentration of 5 μg/ml. After either 5, 10, or 20 min at 30° deoxyribonuclease (Worthington Biochemical Co.) was added to give a concentration of 1 μg/ml. Aliquots were then withdrawn into tubes containing medium at 37°. The amount of medium into which each aliquot was withdrawn was chosen so as to allow the cells to remain in exponential growth during the incubation period. After various lengths of time these sub-cultures were chilled in an ice-bath.

Samples were assayed for total cells, maltose-utilizing transformants and streptomycin-resistant transformants by means of colony counts in dilution tubes containing selective media and antibody[2,6]. The contents of the tubes were then centrifuged and resuspended in 5 ml Tris buffer, 0.05 M, pH 7.3. Then 0.2 ml were removed for protein determination with the Folin-Ciocalteau phenol reagent (Amend Chemical Co.) according to the procedure of Lowry *et al.*[7] and following the suggestions of Oyama and Eagle[8]. The suspension was then centrifuged and the cells were assayed for amylomaltase content.

Measurement of amylomaltase depended on the specific oxidation of glucose, one of the products of its action on maltose, by glucose oxidase to give H_2O_2 which in the presence of peroxidase reacted with *o*-dianisidine to give a colored product*. Cells to be assayed were taken up in 0.2 ml Tris buffer, 0.05 M, pH 7.3, containing 0.02 M NaF, 0.003 M glutathione, 0.1 M NaCl, and 0.07% sodium deoxycholate. Addition of glutathione was essential for the preservation of enzyme activity; fluoride inhibited further metabolism of glucose; NaCl and deoxycholate were necessary for lysis of the cells. On incubation at 37° for 10 min the cells were lysed. After chilling the lysates, 0.1 ml of 6 % maltose (twice recrystallized from alcohol) was added, and the mixtures were incubated for 2 h at 37°. To end the reaction the samples were heated for 5 min at 100°. Glutathione was destroyed by the addition of 0.05 ml 1 % H_2O_2 which in turn was destroyed by subsequently treating with 0.05 ml of 0.02 % catalase (lyophilized preparation, Nutritional Biochemicals). Heating again for 5 min at 100° destroyed the catalase. To the samples were added 3 ml of a solution containing 3.75 mg glucose oxidase (Worthington), 0.15 mg horseradish peroxidase (Worthington), and 0.3 mg *o*-dianisidine (Eastman Organic Chemicals) in Tris buffer, 0.05 *M*, pH 8.5. At this pH interference by maltase present in the glucose oxidase preparation was eliminated. After 15 min at room temperature 0.05 ml of 4 *N* HCl was added to end the reaction and stabilize the color. Coagulated protein was removed by centrifugation and the O.D. of the clear supernates was read at 420 mμ in a Beckman spectrophotometer. The proportionality of color to the amount of amylomaltase, even in the presence of large amounts of lysed amylomaltase-deficient cells, is shown in Fig. 1. Amylomaltase activity will be expressed in terms of a standard unit defined as that amount which produces 1 mμmole of glucose/min.

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* This procedure for measuring glucose and the necessary reagents are marketed by the Worthington Biochemical Company, Freehold, New Jersey, under the trade-name "Glucostat".

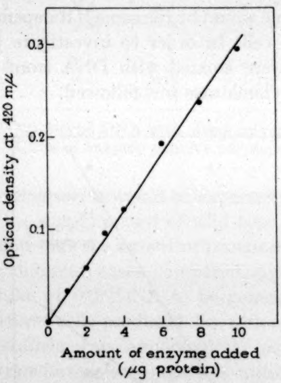

Fig. 1. Proportionality between amount of amylomaltase and color produced in the assay reaction. The indicated amounts of a purified amylomaltase preparation were added to mixtures of NaF, NaCl, glutathione, sodium deoxycholate, and cells of the negative strain, Mc, equivalent to 0.1 mg protein, in 0.2 ml Tris buffer. Lysis of the cells occurred on incubation for 10 min at 37°. Maltose was added and incubation was continued for 1 h. Samples were assayed for glucose as described in the text. The value found for the maltose blank, 0.010, was subtracted from the O.D. readings.

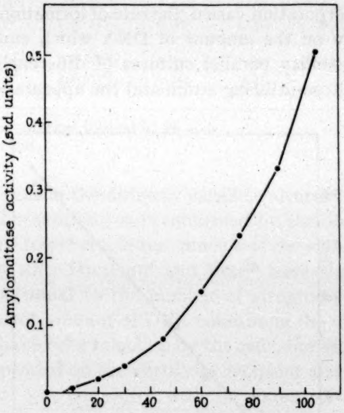

Fig. 2. Appearance of amylomaltase activity in a transformed culture of a negative strain. A suspension of Mg cells was treated with wild-type DNA for 5 min at 30°. After treatment with deoxyribonuclease for 1 min, samples were diluted into fresh medium at 37° and incubated for various lengths of time. Amylomaltase activity in the samples was determined as described in the text.

RESULTS

Soon after a brief exposure to DNA from the wild strain amylomaltase, activity appeared in a transformed culture of a mutant strain. This is shown in Fig. 2 for the case of strain Mg treated for 5 min with DNA from strain R6S. Although there appears to be a lag of about 6 min, within 10 min enzyme was detectable and the rate of formation augmented with growth of the culture.

When the increase in amylomaltase is examined as a function of the increase in total protein, a linear relationship is observed (Fig. 3). At first, protein increases to the extent of 20 % without the appearance of any enzyme; this increase in protein corresponds to a lag in time of about 6 min. By 10 min, however, and thereafter, amylomaltase is synthesized at its maximal rate, as a constant proportion of new protein.

The increasing rate of enzyme formation, clearly evident by 40 min (Fig. 2) parallels the increasing rate of protein synthesis in the exponentially growing culture (Fig. 3). This correspondence indicates that at least by this time the newly introduced genetic factor is multiplying as rapidly as the genetic factors responsible for protein synthesis in general, that is, the host genome.

It has been reported[2] that cells of different mutant strains are genetically transformed by R6S DNA to maltose utilization with different frequencies even when transformation to streptomycin-resistance occurs with the same frequency in the different strains. It might be expected that although the extent of permanent genetic incorporation varied, the rate of formation of enzyme would be the same if it depended only on the amount of DNA which entered the cell. In order to investigate this possibility parallel cultures of different strains were treated with DNA from the maltose-utilizing strain and the appearance of amylomaltase was followed.

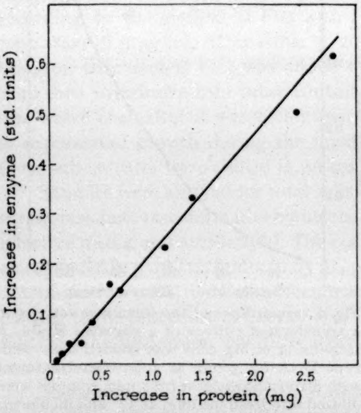

Fig. 3. Formation of amylomaltase with respect to protein synthesis in a transformed culture. Same experiment as in Fig. 2. Amylomaltase and protein were determined as described in the text. Prior to incubation the samples contained 0.27 mg protein and no detectible amylomaltase activity.

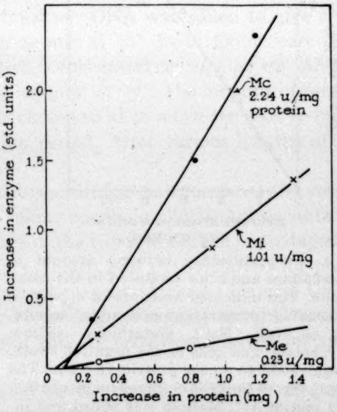

Fig. 4. Rates of formation of amylomaltse in transformed cultures of different mutant strains. Cultures of Mc, Mi, and Me, frozen at the same stage of growth, were thawed and treated with wild-type DNA for 20 min at 30°. After treatment with deoxyribonuclease for 1 min, samples were diluted into fresh medium and incubated 0, 30, 60, and 90 min at 37°. Initially, the protein content of each culture was about 0.3 mg, and no amylomaltase was detectible.

Fig. 4 presents the results obtained in comparing strains Mc, Mi, and Me which are permanently transformed to maltose-utilization at frequencies in the ratios of 10:4:1. In all cases the kinetics of enzyme formation was the same: after a brief delay amylomaltase was produced as a constant proportion of total protein. It is clear, however, that the rates were proportional to the extent of permanent transformation of the maltose character. Similar results were obtained in comparing strains Mg and Md (Fig. 5). Table I summarizes these results.

TABLE I

RELATIONSHIP OF THE RATE OF FORMATION OF ENZYME TO THE EXTENT OF PERMANENT TRANSFORMATION

Expt.	*Recipient strain*	*DNA donor**	*Total cells*	*Transformants to:*		*Rate of amylo-maltase formation***	*Relative extent of transformation to mal+*	*Relative rate of enzyme formation*
				Streptomycin resistance	*Maltose utilization*			
1	Mc	++	$1.5 \cdot 10^9$	$3.4 \cdot 10^7$	$5.5 \cdot 10^7$	2.24	1.00	1.00
	Mi	++	$1.7 \cdot 10^9$	$2.7 \cdot 10^7$	$2.1 \cdot 10^7$	1.01	0.38	0.45
	Me	++	$1.5 \cdot 10^9$	$3.3 \cdot 10^7$	$3.3 \cdot 10^6$	0.23	0.06	0.10
2	Mg	++	$3.5 \cdot 10^9$	$2.9 \cdot 10^7$	$4.6 \cdot 10^7$	2.47	1.00	1.00
	Md	++	$3.1 \cdot 10^9$	$2.8 \cdot 10^7$	$1.8 \cdot 10^7$	0.98	0.39	0.40
3	Mc	++	$7.2 \cdot 10^8$	$1.1 \cdot 10^7$	$1.6 \cdot 10^7$	1.29	1.00	1.00
	Mc	MfS	$7.0 \cdot 10^8$	$1.0 \cdot 10^7$	$4.6 \cdot 10^6$	0.29	0.29	0.23

* Wild-type DNA with streptomycin-resistance marker indicated as ++.
** Units of enzyme activity per mg of new protein.

A priori it would be expected that the cells in the different parallel cultures took up the same amount of wild-type DNA. This supposition is confirmed by the observation that the extent of transformation to streptomycin-resistance was the same in the different cases compared, for LERMAN AND TOLMACH[9] and FOX[10] have shown uptake of [^{32}P]DNA to be generally proportional to the number of streptomycin-resistant cells produced. So, although the same amount of DNA containing the wild-type allele of the amylomaltase locus must have been taken up by the cells, the extent of transformation to maltose-utilization depended on the particular recipient and the rate of formation of enzyme from the start was correlated with the extent of this permanent genetic change.

Amylomaltase-deficient strains in certain cases can be crossed to produce maltose-utilizing cells[2]. The appearance of enzyme was followed in one such cross in which Mc was the recipient and MfS the donor of the DNA. Fig. 6 shows that enzyme synthesis was initiated as rapidly with mutant as with wild-type DNA. As in the latter case, the amount of enzyme formed was proportional to total protein synthesized. However, the rate of enzyme formation was reduced, corresponding to the reduced extent of permanent transformation to maltose-utilization, which was in this case occasioned by the necessity for recombination between closely linked sites. Unless this correspondence is fortuitous, it seems probable that the genetic elements of strains Mc and Mf do not complement each other when on separate structures. Observations in which complementation of function occurs between closely-linked sites apparently governing individual enzymes[11,12] have been rare and may not refer to sites on homologous DNA molecules. Therefore, it is most likely that some form of recombination between different, homologous genetic structures must have occurred very early in order to allow amylomaltase to be synthesized at its maximal rate by 15 min after DNA treatment.

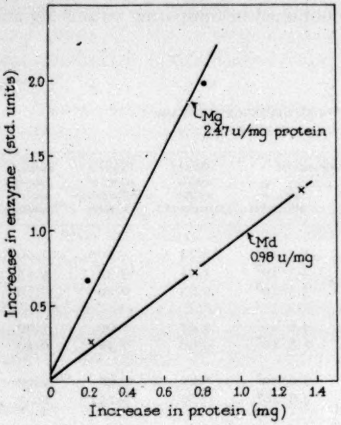

Fig. 5. Rates of formation of amylomaltase in transformed cultures of strains Mg and Md. Procedure was similar to the experiment depicted in Fig. 4. Initially, the cultures contained 0.4 mg of protein.

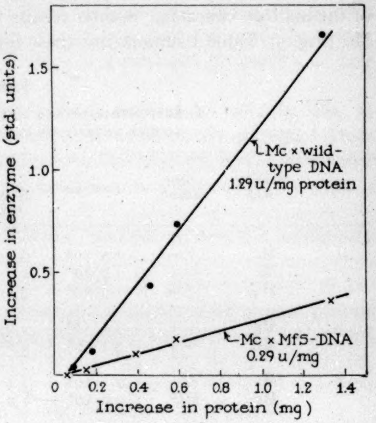

Fig. 6. Formation of amylomaltase after transformation with DNA from wild-type and mutant strains. A suspension of Mc cells was divided into two portions which were respectively treated with equivalent amounts of DNA from strains R6S and MfS for 10 min at 30°. After treatment with deoxyribonuclease for 1 min, samples of each portion were diluted into fresh medium and incubated at 37° for 0, 15, 30, 45, 60, and 90 min. Initially, 0.25 mg protein was present and no amylomaltase was detectable.

DISCUSSION

Amylomaltase is rapidly formed subsequent to the introduction of DNA conveying the genetic capacity to produce the enzyme into cells previously unable to synthesize it. Within 6 min its synthesis has begun and within ten minutes is proceeding at a maximal rate—as a constant proportion of protein synthesis. However, synthesis of the new protein appears to involve the prior integration of the genetic factor carried by the donor DNA. When strains which show different frequencies of permanent transformation of the maltose character are transformed, the rates of enzyme formation are proportional to the extent of this transformation, even though the same amount of donor DNA was taken up by the cells in the different cases. Thus, "free" DNA in the cell does not seem to give rise to enzyme synthesis. It appears that some sort of permanent integration must be necessary for synthesis to occur. Such integration must occur early inasmuch as the early attainment of the constant rate of enzyme formation with protein synthesis precludes the possibility of any long delay in the integration of the genetic factor. Furthermore, the persistence of this rate of enzyme formation indicates that there is no loss of the new factor once it is integrated; that is, no temporary transformation has occurred.

The proportionality between permanent transformation and amylomaltase synthesis holds as well for crosses between two mutant strains. Here enzyme synthesis demands not only integration of the transforming DNA molecule into an organized, functional state, but, presumably before that, recombination (either direct or dependent on replication) of genetic factors in two molecules of DNA. Yet the rapidity of onset and subsequent constancy of enzyme production with respect to protein formation attest that all recombination must have occurred very soon after DNA treatment and that "recombined" DNA is being reproduced not long thereafter.

The various results present a consistent pattern underlying the development of enzyme activity after introduction of DNA into the cell. (a) Following entry into the cell, free DNA must be integrated before it gives rise to enzyme synthesis. (b) Such integration is rapid since enzyme synthesis begins less than 10 min after DNA treatment. (c) Recombination between parts of donor and host DNA molecules to produce a new phenotype also must occur within this early period. (d) Replication of the new genetic factor must occur very soon since the constant rate of enzyme synthesis with respect to protein appears to be reached shortly thereafter; hence, any integration necessary for replication has already occurred. Thus, three different events–integration with respect to phenotypic expression, genetic recombination, and integration prerequisite to replication–all seem to occur within the same brief period. They may well be manifestations of a single process– of incorporation into the genome.

The above conclusions depend on the promptness of phenotypic expression found with the amylomaltase system. Other characters such as pyrimidine-independence, sucrose utilization, and sulfonamide-resistance may also be expressed promptly, since transformants to these traits will survive early selection. However, studies of the expression of streptomycin-resistance in pneumococcus have shown that this character does not manifest itself until, on the average, sixty minutes after incorporation of the DNA[13, 14]. Perhaps streptomycin-resistance does not involve simply the presence of several molecules of enzyme, but rather more substantial changes, such as in the composition of the cell wall which would require several generations in order to have altered its dominant motif. Or looked at in another way, streptomycin-sensitivity may result from the presence of certain substances which would have to be diluted out of the cells by division before any cells could become resistant. These possibilities would be consistent with the finding that in diploid cells of *E. coli* streptomycin-resistance behaves as a recessive character[15]. (However, evidence has been presented that the contrary is true for pneumococcus[16].)

That DNA must be integrated in permanent fashion before it can exercise its function was an unexpected finding. Precisely what the nature of this integration is remains undetermined. It may be that DNA can act only when incorporated into a nuclear organization, or it may simply have to be integrated to protect it from degradation. The inability of DNA incorporated but not integrated in the cell to produce phenotypic expression, differentiates transformation from transduction in *Salmonella* where the occurrence of "abortive transduction" indicates an activity of genetic material not permanently incorporated into the host genome[17, 18]. It may be that the transduced genes are in an adequate state of organization to function, that is, more highly organized than free DNA. This is suggested by the fact that a transducing particle usually contains a number of linked genes and may thus constitute an appreciable segment of a bacterial chromosome. Such an organized segment may be able to persist indefinitely as a genetic structure even though not replicated.

One of the conclusions stemming from the present work is that the genetic factor in the transforming DNA as soon as it is integrated begins itself to be reproduced by the recipient cell. Replication of the new factor as manifested by increasing enzyme formation occurs within 40 min after incorporation, A prior estimate of the time of earliest replication as 55 min[19] is based on the multiplication of streptomycin-resistant colony-forming units which is observed within the second hour after introduction of DNA[19, 20].

It is of interest to compare the manner in which amylomaltase is synthesized subsequent to transformation with specific protein synthesis elicited in other systems, for there seems to be a general pattern governing synthesis of new protein. One of the first such systems studied was induction of β-galactosidase in *E. coli* upon addition of an inducer substance[21]. Synthesis of the β-galactosidase begins at once and continues as a constant proportion of total protein synthesis. Again in *E. coli*, the release of a constitutive enzyme, ornithine carbamylase, from repression by lowering the arginine concentration also results in prompt appearance of the enzyme, its production being proportional to total synthesis[22].

Most similar to the present one is the system studied by PARDEE, JACOB AND MONOD[23] in which β-galactosidase is produced in *E. coli* cells which have undergone conjugation, for here also the eliciting mechanism is the introduction of genetic material, albeit presumably in a more organized, chromosomal, form. In this case the enzyme is produced very quickly after a brief period of conjugation and at a linear rate with time. In another case in which genetic material is introduced into a bacterial cell, when T-even bacteriophages infect cells of *E. coli*, an enzyme essential for the growth of the virus, deoxycytidylate hydroxymethylase, is produced a few minutes after infection[24].

Observations from these quite different systems are in agreement that (a) protein synthesis is a very rapid process, even when it must of necessity be *de novo* due to prior absence of a genetic factor, and (b) increase in a particular protein is proportional to increase in total protein. The level of an enzyme appears in general to play no direct role in governing its synthesis. Rather, if it is being formed, its rate of formation seems to be part of a fixed order in the cell.

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STUDIES ON THE BIOSYNTHESIS OF 5-RIBOSYLURACIL PHOSPHATE IN *NEUROSPORA CRASSA* 36601

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SUMMARY

(1) While the pyrimidine portion of 5-ribosyluracil phosphate has been found to be derived from orotic acid in yeast, it has been found to be derived largely from cytidine of the medium in the pyrimidine requiring mutant Neurospora, *Neurospora crassa* 36601.

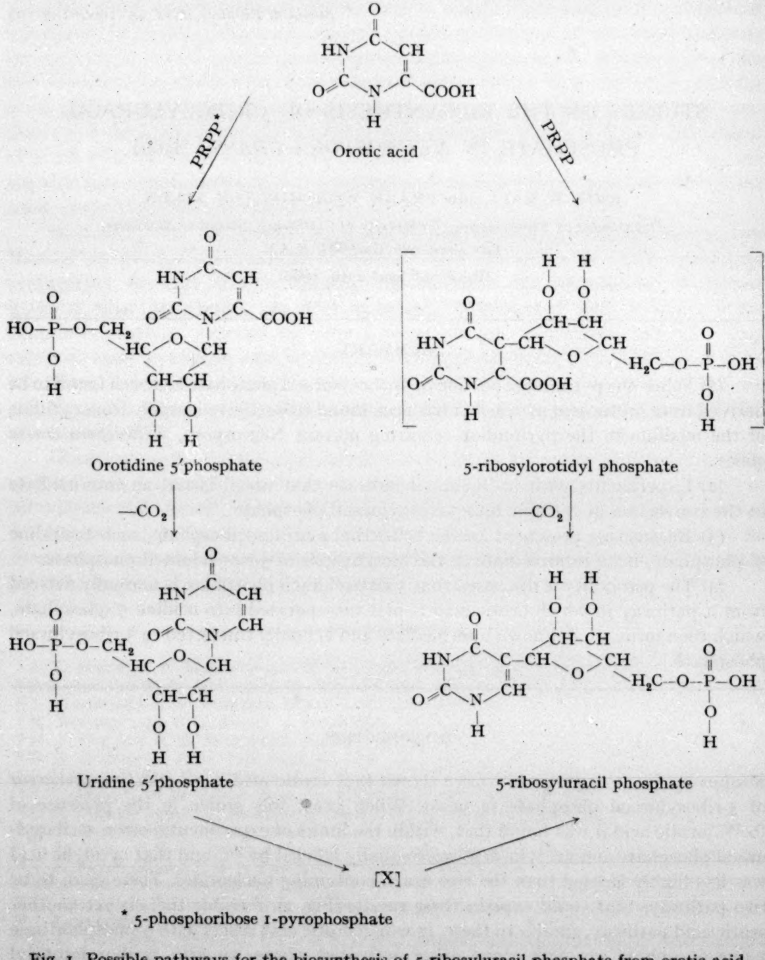
(2) Experiments with [2-^{14}C]uracil indicate that uracil is not an intermediate in the conversion of cytidine into 5-ribosyluracil phosphate.

(3) Reasons are presented for the belief that a uridine phosphate, such as uridine 5′-phosphate, is an intermediate in the biosynthesis of 5-ribosyluracil phosphate.

(4) The possibility is discussed that 5-ribosyluracil phosphate is normally derived from a pathway in which orotic acid is first incorporated into uridine 5′-phosphate, which then forms an unknown intermediate and is finally converted to 5-ribosyluracil phosphate.

INTRODUCTION

Studies by Hall and Allen[1] have shown that orotic acid is an effective precursor of 5-ribosyluracil phosphate in yeast. When yeast was grown in the presence of [6-^{14}C]orotic acid it was found that, within the limits of experimental error, 5-ribosyluracil phosphate and uridylic acid were equally labeled by ^{14}C and that cytidylic acid was less highly labeled than the two uracil-containing nucleotides. There seem to be two pathways that could explain these results (Fig. 1). Possibly there is yet another orotic acid pathway, similar to these, in which orotic acid reacts with 5-phosphoribose 1-pyrophosphate, or with some other activated ribose, to give 5-ribosylorotidyl phosphate, which is an isomer of orotidine 5′-phosphate, in which the ribose is linked to the carbon atom of position 5 of the orotate molecule, rather than with the nitrogen atom of position 3. This compound could then be directly decarboxylated to give 5-ribosyluracil phosphate, just as orotidine 5′-phosphate yields uridine 5′-phosphate upon decarboxylation. The other possibility is that 5-ribosyluracil phosphate is derived from some nucleotide intermediate further along the orotic acid pathway. Cytidylic acid is ruled out by the pattern of labeling in the experiment on yeast,


Orotic acid
PRPP*
PRPP
Orotidine 5′phosphate
5-ribosylorotidyl phosphate
—CO₂
—CO₂
Uridine 5′phosphate
5-ribosyluracil phosphate
[X]

* 5-phosphoribose 1-pyrophosphate

Fig. 1. Possible pathways for the biosynthesis of 5-ribosyluracil phosphate from orotic acid.

because it is unlikely that a compound could be derived from another compound in the metabolic sequence that is less highly labeled. The data, however, indicate that 5-ribosyluracil phosphate could be derived from uridylic acid, and that perhaps it is derived exclusively from this compound.

To examine these two possibilities it was decided to determine the origin of the pyrimidine of 5-ribosyluracil phosphate in a mutant organism which can not utilize orotate for the biosynthesis of its pyrimidine nucleotides. *Neurospora crassa* 36601 was selected for this purpose. This fungus, which was first described by MITCHELL, HOULAHAN AND NYC[2], requires pyrimidines for growth and grows best on nucleosides, such as cytidine. Orotidine and orotic acid accumulate in the culture medium, so that the fungus appears to lack the decarboxylase which converts orotidine 5′-phosphate to uridine 5′-phosphate. This mutant does not to any extent incorporate orotic acid into the pyrimidine nucleotides uridylic acid and cytidylic acid. If orotic acid were to be incorporated into 5-ribosyluracil phosphate, the existence of an orotic acid pathway parallel to the one utilized for the synthesis of the principal pyrimidine nucleotides would be demonstrated. If, on the other hand, orotic acid were not to be incorporated into 5-ribosyluracil phosphate, then the problem of its origin from other precursors would arise. This latter, is in fact, what has been observed in this study, so that it seems probable that the pyrimidine of 5-ribosyluracil phosphate is derived from the cytidine furnished in the medium. The pathway by which this conversion is accomplished is the subject of this research.

EXPERIMENTAL

Materials

Neurospora crassa 36601 was obtained as a dry pellet of lyophilized mycelia in a sealed vial from the American Type Culture Collection, 2112 M Street, N.W., Washington 7, D.C. The pellet was put into a sterile test tube which contained about 20 ml of culture medium and was allowed to grow at room temperature. Growth was apparent in about 3 days and portions of the mycelia were than transferred to agar slants and maintained as stock cultures. The purity of the strain was periodically checked by transferring a portion of the mold to a test tube containing the same medium on which it had been grown, with the exception that cytidine was omitted. The mutant requires pyrimidines for growth, and in no case did growth occur on a medium deficient in these. This procedure guarded against the possibility of back-mutation and of contamination of the culture by organisms that did not require cytidine.

Culture medium for Neurospora crassa *36601*

The culture medium for this mutant Neurospora is the one described by BEADLE AND TATUM[3] and is based on a salt mixture developed by FRIES[4] designated No. 3. To this medium 20 units penicillin/ml and 40 μg streptomycin/ml[5] are added to suppress the growth of any bacteria that might gain entrance after autoclaving.

The medium was made up to 5.5 l and placed in a 20-l pyrex bottle. The mouth of the bottle was stoppered with a cotton plug and the bottle was autoclaved at 100° for 1 h. This low-pressure autoclaving was sufficient to sterilize the medium and did not induce the formation of sediment or cause caramelization of the sugar.

The medium for the seed cultures was of the same composition as that for the batch cultures. This medium was poured into test tubes stoppered with cotton plugs and autoclaved. The agar slants were prepared from the same medium with agar added to a concentration of 1.5 % (w/v).

Radioactive compounds

[2-^{14}C]Uracil and [6-^{14}C]orotic acid were purchased from the Research Specialties Company of Richmond, Calif.

[2-^{14}C]Cytidine was obtained from the Schwarz Laboratories of Mount Vernon, N.Y.

A small amount of contaminating uracil was removed from the orotic acid by chromatography on a Dowex-1-acetate column. The purity of the cytidine and of the uracil was examined by paper chromatography in butanol–water. The uracil appeared to be pure, but the cytidine was contaminated by a trace of material which was believed to be uridine. It was thought that the amount of this contaminant was too small to interfere with the experiments.

Methods

11 mg [6-^{14}C] orotic acid containing $27 \cdot 10^6$ counts/min was added to 5.5 l of the culture medium. The medium was inoculated with a seed culture containing 50–100 mg *Neurospora crassa* 36601 and the bottle of medium, of approx. 20-l capacity, was placed on its side on a rotator. This device caused the bottle to rotate about its long axis and stirred and aerated the medium. After 2 days the mold was harvested by pouring the medium containing it through a layer of cheese cloth. The fungus was then squeezed dry and weighed. About 10 g was usually obtained. This was about one-third of the maximum weight obtainable when longer growth periods were used, a fact which indicated that the mold was still growing vigorously at the time of harvest. The fungus was placed in a chilled Waring blendor, which contained 100 ml of a cold solution of 8 parts ethanol to 2 parts of a 10-% solution of perchloric acid, and it was blended for 10 min. The resulting homogenate was centrifuged and the precipitate was washed with 10 % perchloric acid to remove additional acid-soluble material. The homogenized residue was neutralized with solid pellets of potassium hydroxide and 100 ml 1 *N* potassium hydroxide was added. The mixture was thoroughly stirred and allowed to stand at room temperature for 24 h to hydrolyze the nucleic acids to mononucleotides. This procedure, which is a modification of that of SCHMIDT AND THANNHAUSER[6], was adopted after two attempts to extract the nucleic acid of this organism by the phenol method of LITTAUER AND EISENBERG[7] had failed to give satisfactory yields. Treatment of a commercial sample of cytidylic acid in a similar manner showed that hydrolysis with potassium hydroxide under these conditions does not produce significant deamination of cytidylic acid. The potassium hydroxide of the Neurospora homogenate was neutralized by the addition of concentrated acetic acid and centrifuged. The residue was washed twice with 67 % ethanol. A cloudy supernatant was obtained from the original centrifugation, and this was poured into two volumes absolute alcohol, which precipitated a pinkish-white, curdy material. This step was introduced when it was found that the cloudy material interfered with further purification and was not soluble in lipid solvents or precipitated by extremes of pH. The precipitate was removed by centrifugation and washed with 67 % ethanol. The

alcoholic solution containing the nucleotides was reduced in volume on a rotary evaporator and desalted on a charcoal column[8].

After adsorption on charcoal and elution, the solution containing the nucleotide was dried on a flash evaporator to remove ammonia and alcohol. A drop of acetic acid was added to reduce the pH to approx. 6, and the material was washed on to the surface of a Dowex-1-acetate column. The system of column chromatography used to fractionate the nucleotides was that devised by COHEN[9] and described by LANE AND BUTLER[10]. This system resulted in excellent separation of 5-ribosyluracil phosphate from uridylic acid. The elution of fractions from this column was recorded by the recording unit of a GME automatic fraction collector. The fractions containing the nucleotides were collected and desalted on charcoal. The solution of desalted nucleotides was reduced in volume and placed on Whatman No. 1 chromatography paper to be purified by ascending chromatography in either isopropanol–hydrochloric acid–water (170:20:39)[11] or in isopropanol–acetic acid–water (60:30:10)[12]. Approx. 200 μg/cm was applied to the paper and the chromatograms were developed overnight. The first solvent required but one submission, but, when the second was used, two submissions were necessary. The chromatograms were photographed in u.v. light and the areas that absorbed were cut out and eluted. Spectral ratios of the purified nucleotide solutions were calculated.

Aliquots of the nucleotide solutions were now plated in duplicate or triplicate, dried in a vacuum desiccator under an infra-red lamp, and counted three times on the automatic gas flow counter. The counts/min were averaged and background subtracted, and the specific activities in counts/min/mmole were calculated.

Procedures for the subsequent experiments were essentially the same as that described for the incorporation of orotic acid into the nucleotides of the mutant Neurospora. Different substances containing the ^{14}C label were added to the medium, but the conditions of growth and the isolation of the nucleotides were largely the same in other respects. When labeled cytidine was used, it was added to the medium as 0.37 mmoles cytidine with an activity of $7.0 \cdot 10^7$ counts/min/mmole. In the experiment designed to determine the role of uracil in the biosynthesis of 5-ribosyluracil phosphate, 0.17 mmoles uracil containing $2.1 \cdot 10^8$ counts/min/mmole, was added to the cytidine supplemented medium.

In order to determine the dilution of uracil before its incorporation into the nucleotides in this last experiment, the free uracil of the cell was isolated from the Neurospora homogenate by the following method. The combined alcohol–perchloric acid supernatant solution from the Neurospora homogenate and the 10 % perchloric acid wash were neutralized with potassium hydroxide. 1.5 g of barium acetate was added to this solution, which was about 300 ml in volume, and the solution was then adjusted to pH 9 with barium hydroxide. The precipitate of potassium perchlorate and the barium salts of the nucleotides was centrifuged and washed with 100 ml water. The supernatant solution was evaporated to a volume of approx. 100 ml on the rotary evaporator and desalted on charcoal. An aliquot of the resulting solution containing approx. 3 mg material was placed across a 1-cm wide strip of Whatman No. 1 paper and twice submitted to ascending chromatography in butanol saturated with water[13]. The material separated into several bands. Scanning with a radioactivity counter showed that one of these, which had moved in about the position expected of uracil in this solvent, was highly radioactive. This band was cut out, eluted, and submitted to chromatography in isopropanol–hydrochloric acid–water, to remove any traces of contaminating material. The material moved at a rate identical with that of a reference spot of uracil on the same sheet of paper. The area containing the radioactive-uracil was cut out and eluted and the spectral ratios were determined, and the pyrimidine was plated and counted as described earlier.

TABLE I

SPECIFIC ACTIVITIES OF NUCLEOTIDES DERIVED FROM THE NUCLEIC ACIDS OF *Neurospora crassa* 36601 GROWN ON VARIOUS ^{14}C LABELED PRECURSORS

	Specific activity, counts/min/mmole			
	Precursor			
	Orotic Acid	*Cytidine 1*	*Cytidine 2*	*Uracil*
Precursor	$3.9 \cdot 10^8$	$7.0 \cdot 10^7$	$7.0 \cdot 10^7$	$2.1 \cdot 10^8$
Cytidylic acid	$1.8 \cdot 10^6$	$7.0 \cdot 10^7$	$6.4 \cdot 10^7$	$1.2 \cdot 10^7$
Uridylic acid	$2.3 \cdot 10^6$	$6.8 \cdot 10^7$	$6.2 \cdot 10^7$	$2.0 \cdot 10^7$
5-Ribosyluracil phosphate	$2.3 \cdot 10^6$	$5.8 \cdot 10^7$	$5.1 \cdot 10^7$	$1.7 \cdot 10^7$
Adenylic	$3.5 \cdot 10^4$	$1.1 \cdot 10^6$	$1.0 \cdot 10^6$	$1.0 \cdot 10^5$
Guanylic acid	$2.8 \cdot 10^4$	—	$6.3 \cdot 10^5$	$8.0 \cdot 10^4$
Cellular uracil	—	—	—	$5.1 \cdot 10^7$

TABLE II

THE UTILIZATION OF VARIOUS ^{14}C LABELED COMPOUNDS AS PRECURSORS FOR NUCLEIC ACID NUCLEOTIDES IN *Neurospora crassa* 36601

	Per cent nucleotide derived from precursor			
	Precursor			
	Orotic acid	*Cytidine 1*	*Cytidine 2*	*Uracil*
Cytidylic acid	0.5	100	91	5.7
Uridylic acid	0.6	97	89	9.5
5-Ribosyluracil phosphate	0.6	83	73	8.1
Adenylic acid	0.009	1.6	1.4	0.05
Guanylic acid	0.007	—	0.9	0.04
Cellular uracil	—	—	—	24

RESULTS AND DISCUSSION

The specific activities of the nucleotides isolated from *Neurospora crassa* 36601, after this fungus had been grown in the presence of various labeled pyrimidine precursors, are given in Table I. The efficiency of each precursor for the formation of the pyrimidine nucleotides is more readily seen in Table II, in which the data are presented as the % nucleotide derived from each precursor, obtained by dividing the specific activity of the isolated nucleotide by that of its precursor and multiplying by 100. Data for the purine nucleotides are included as a measure of the degradation and non-specific incorporation of the labeled compound into nucleic acids.

It can be seen from Table II that orotic acid produces very little labeling of any of the pyrimidine nucleotides. Because the orotic acid had been purified, it was unlikely that any pyrimidine precursor would be present at levels that would produce the labeling observed. It was therefore decided that the slight incorporation of ^{14}C into the pyrimidine nucleotides represented a leak in the block of the orotic acid pathway. It will be seen that the pattern of incorporation produced by this precursor is identical with that reported earlier for yeast[1]. The possibility that 5-ribosyluracil phosphate, unlike the major pyrimidine nucleotides, could be derived from orotate in this organism is excluded by this experiment. The most probable source, therefore of the pyrimidine of this compound is the cytidine furnished in the medium. When the Neurospora was grown in the presence of ^{14}C-labeled cytidine, it was found that this nucleoside was an effective precursor for the pyrimidine of all three pyrimidine nucleotides. This was the expected result, but it raised the question of what is the sequence of metabolic transformations that are utilized by the organism to convert a nucleoside, with the ribose moiety attached to a nitrogen atom in the 3 position of the ring, into a nucleotide, in which the ribose is attached to a carbon atom in the 5 position. It seems unlikely that 5-ribosyluracil phosphate is derived directly from cytidine, through some intermediate such as 3,5-diribosylcytosine, because the specific

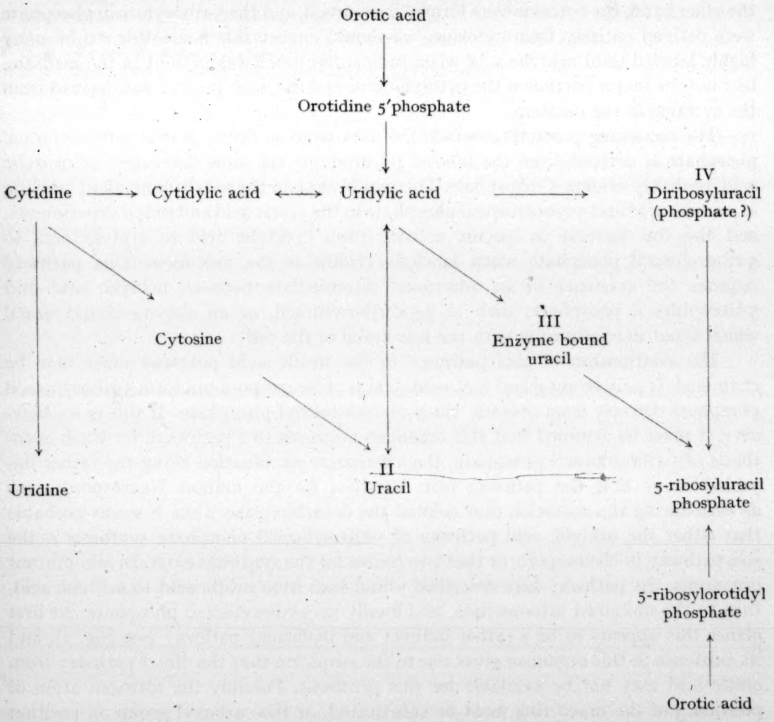

Orotic acid
Orotidine 5′phosphate
Cytidine
Cytidylic acid
Uridylic acid
IV
Diribosyluracil (phosphate ?)
Cytosine
III
Enzyme bound uracil
Uridine
II
Uracil
5-ribosyluracil phosphate
5-ribosylorotidyl phosphate
Orotic acid

Fig. 2. Some possible intermediates in the biosynthesis of 5-ribosyluracil phosphate.

activity of 5-ribosyluracil phosphate is even less than that of uridylic acid, instead of being comparable to that of cytidylic acid, as would be expected if radioactive-cytidine were the precursor. A second possibility is that cytidine is first converted to a free pyrimidine, which is then conjugated with ribose to form 5-ribosyluracil phosphate (Fig. 2). To examine this possibility, the Neurospora was grown on a cytidine supplemented medium to which [2-^{14}C]uracil had been added. It was again found that 5-ribosyluracil phosphate was less highly labeled than uridylic acid, although its specific acitivity was higher than that of cytidylic acid, as it was in the orotic acid experiments. If uracil were an intermediate in the biosynthesis of 5-ribosyluracil phosphate, we should expect this nucleotide to be much more, rather than less highly labeled than uridylic acid. The fact that the free uracil isolated from the Neurospora homogenate was more than two and a half times as radioactive as 5-ribosyluracil phosphate showed that this nucleotide was not derived principally from uracil. It is possible that cytosine, and not uracil, is the free pyrimidine intermediate. This seems unlikely, however. If cytosine is derived from cytidylic acid, it is clear that it cannot be the precursor of 5-ribosyluracil phosphate in the uracil or orotic acid experiments in which cytidylic acid was less highly labeled than the uracil nucleotides were. If, on the other hand, the cytosine were formed from uracil, and the 5-ribosyluracil phosphate were derived entirely from cytosine, we should expect this nucleotide, to be more highly labeled than uridylic acid when radioactive uracil was present in the medium, because the major portion of the cytidylic acid and the uridylic acid was derived from the cytidine in the medium.

The remaining possibility, which the data seem to favor, is that 5-ribosyluracil phosphate is derived from the labeled pyrimidines via some derivative of uridylic acid, probably uridine 5'-phosphate. This would explain the nearly equivalent labeling of uridylic acid and 5-ribosyluracil phosphate in the orotic acid and uracil experiments, and also the decrease in specific activity from cytidylic acid to uridylic acid to 5-ribosyluracil phosphate when labeled cytidine is the precursor. This pathway requires the existence of an additional intermediate between uridylic acid and 5-ribosyluracil phosphate, such as 3,5-diribosyluracil, or an enzyme-bound uracil which is not in equilibrium with the free uracil of the cell.

The relationship of this pathway to the orotic acid pathway must now be examined. It may be supposed that wild strains of Neurospora can form 5-ribosyluracil phosphate directly from orotate, via 5-ribosylorotidyl phosphate. If this is so, however, it must be assumed that this organism possesses two pathways for the biosynthesis of 5-ribosyluracil phosphate, the alternative explanation being the rather improbable one that the pathway just described for the mutant Neurospora arose *de novo* during the mutation that deleted the decarboxylase. Thus it seems probable that either the uridylic acid pathway of 5-ribosyluracil phosphate synthesis is the sole pathway in Neurospora, or that two routes for the synthesis exist. In non-mutant organisms, the pathway here described would lead from orotic acid to uridylic acid, then to the unknown intermediate, and finally to 5-ribosyluracil phosphate. At first glance this appears to be a rather indirect and inefficient pathway (see Fig. 1), and its existence in this organism gives rise to the suspicion that the direct pathway from orotic acid may not be available for this synthesis. Possibly the nitrogen atom of position 3 of the uracil ring must be substituted, or the carboxyl group on position 4 removed, before the carbon atom at position 5 can react with a ribosyl compound to form a 5-ribosyluracil derivative. This is highly speculative, however. All that can be said with confidence is that the data indicate that this mutant of neurospora is able to utilize cytidine and uracil for the synthesis of 5-ribosyluracil phosphate by a pathway which probably involves uridylic acid as an intermediate.

ACKNOWLEDGEMENTS

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ACCUMULATION OF INORGANIC POLYPHOSPHATE IN MUTANTS OF *NEUROSPORA CRASSA*

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SUMMARY

1. 53 strains of *Neurospora crassa*, representing 4 wild types and 40 mutant loci, were examined for accumulation of acid-soluble inorganic polyphosphate. When grown on a limited supply of growth factor, most of the mutants were found to accumulate polyphosphate, though the amounts varied widely.

2. The time sequence of polyphosphate accumulation was studied in shaken cultures of the histidine-requiring strain, C-84. When growth ceased due to exhaustion of the amino acid, the RNA content of the mycelium dropped sharply. Concomitantly, acid-soluble polyphosphate accumulated; the levels of other phosphorus compounds, including the acid-insoluble polyphosphate, were not affected.

3. By the use of ^{32}P it was demonstrated that, in C-84, extensive degradation of RNA occurred upon exhaustion of the histidine. The phosphorus released from RNA was converted to polyphosphate and constituted a major source of phosphorus for polyphosphate accumulation. Similar observations were made with other mutant strains, though the details of the pattern of growth, RNA degradation and polyphosphate accumulation varied from strain to strain.

4. Inhibition of the growth of *Neurospora* strains by withholding a macronutrient or by addition of a metabolic inhibitor did not, in general, induce either RNA degradation or polyphosphate accumulation. However, when mycelium was transferred to medium lacking the carbon source, sucrose, extensive degradation of RNA took place. Acid-soluble polyphosphate and inorganic orthophosphate accumulated under these conditions.

5. Polyphosphate accumulation was inhibited by certain amino acid analogs. In C-84, the histidine analog 2-thiazolealanine was found to inhibit RNA degradation but had no effect on the accumulation of polyphosphate.

6. The relationship of polyphosphate accumulation to the primary genetic block is discussed. The possible role of RNA degradation as the immediate cause of polyphosphate accumulation is considered.

INTRODUCTION

Cultures of genetically blocked strains of micro-organisms have commonly been found to accumulate substances that occur in the wild type in trace amounts only.

Abbreviations: PCA, perchloric acid; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Pi, inorganic orthophosphate; TZA, 2-thiazole alanine.

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Generally, the substances which accumulate are closely related to the metabolic step affected by the mutation. Examples are known, however, of mutant strains which accumulate compounds bearing no obvious relationship to the primary metabolic deficiency[1].

A striking case of this kind was described by HOULAHAN AND MITCHELL[2]. Several nutritional mutants of *Neurospora crassa* were found to accumulate large amounts of inorganic polyphosphate when grown on a limiting supply of their required growth factor. Genetic analysis indicated that this phenomenon was associated with the mutation giving rise to the nutritional requirement rather than with an independent mutant gene. Polyphosphate accumulation was therefore regarded as a secondary, pleiotropic, effect of the nutritional deficiency.

The multiplicity of physiological consequences brought about by a specific gene mutation is an important aspect of gene action but has received relatively little attention. The present paper presents experiments designed to analyze the metabolic pattern through which a block in the synthesis of various amino acids, vitamins, purines or pyrimidines may bring about the accumulation of inorganic polyphosphate.

A preliminary report describing some of this work has been presented[3].

EXPERIMENTAL

Biological methods

Strains of *Neurospora crassa* were obtained from the culture collection of the California Institute of Technology. They were kept on Fries agar minimal medium[4] supplemented with the appropriate growth factors.

Growth experiments were conducted in both stationary and shaken cultures.

Stationary cultures were initiated by inoculating conidia into 20-ml aliquots of Fries medium[4]. Pads were harvested after 4 days at 25°, washed with acetone, dried, weighed and analyzed as described below.

Shaken cultures were grown in a modified Fries medium designed to permit omission of single nutrients without affecting the other constituents. Stock solutions of the following compositions, per liter, were prepared: (A) $MgSO_4 \cdot 7H_2O$, 10 g; $CaCl_2 \cdot 2H_2O$, 2 g; NaCl, 2 g; trace elements[4], 20 ml; Biotin, 60 μg. (B) Sodium potassium tartrate$\cdot 4H_2O$, 230 g. (C) KH_2PO_4, 40 g. (D) NH_4NO_3, 80 g.

Equal volumes of each stock solution were combined and diluted to 10 volumes. 1.5 % sucrose was added as carbon source; other supplements were added as described for the individual experiments. Conidia were harvested from Fries agar medium, filtered through glass wool and introduced into the above medium at a density of 10^5–10^6 conidia per ml. Flat bottom culture flasks of 2.5 l capacity, containing up to 1 l of the suspension, were then placed on a reciprocating shaker and agitated at 100 cycles/min at 25°.

To transfer growing mycelium from one type of medium to another, the mycelium was harvested by gentle filtration, washed several times with water and resuspended in fresh medium. At intervals, aliquots of the culture were removed and the mycelium was filtered off by suction, washed with acetone and dried. The weighed pads were then analyzed.

Growth of *Neurospora* under these conditions was almost logarithmic for the first four doubling times and then slowed down, presumably because of the increasing size of the clumps (Fig. 1). The weights of mycelium in volumetric samples of growing cultures were nevertheless generally reproducible to ± 5 %.

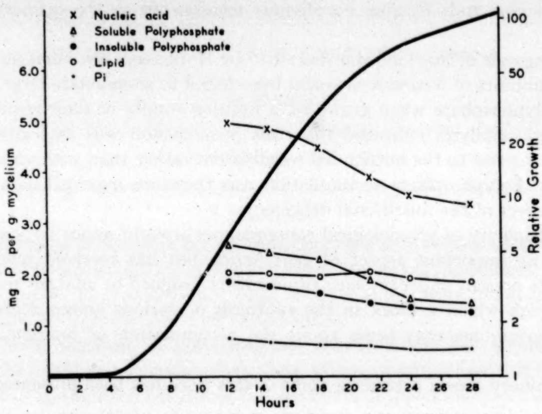

Fig. 1. Growth and phosphorus compounds in a culture of the wild strain, 25a. Conidia were suspended in growth medium at 0 h and the relative mass of mycelium per unit volume of culture is designated as relative growth.

Analytical methods

The analytical methods used in the present study were based upon those developed by others for the fractionation and estimation of phosphorus compounds[5-7], but were simplified to permit the rapid processing of numerous samples.

Fractionation of the mycelium: Samples of acetone-dried mycelium, weighing about 100 mg, were ground with sand and 5 ml cold 0.5 *M* PCA. After standing in the refrigerator for 20 min the mycelium was centrifuged and extracted once more in the same manner. The extracts were pooled and brought to 10 ml.

The residue was then extracted with 5 ml of ethanol at room temperature for 30 min. After centrifugation, 5 ml of ethanol–ethyl ether (3:1) were added to the residue, the mixture was boiled for 1 min, allowed to stand at room temperature for 20 min, and centrifuged again. The two extracts were pooled and brought to 10 ml.

The lipid-free residue was dried by heating in a water bath and then extracted twice with 5-ml portions of hot 0.5 *N* PCA (70°, 15 min). The extracts were pooled and brought to 10 ml.

Estimation of phosphorus compounds: Some or all of the following analytical determinations were performed upon the four fractions.

Cold PCA extract

Inorganic orthophosphate and total phosphorus were determined by ALLEN's method[8]. The ^{32}P contents were measured after extraction into isobutanol[9] and by direct mounting of an aliquot of the extract, respectively.

Acid-labile phosphorus was determined as above after hydrolysis with 1 *N* HCl for 15 min at 100°.

In the early experiments, inorganic polyphosphate extractable with cold PCA ("soluble polyphosphate") was determined by precipitation with Ba^{++} at pH 4.5

(see ref. 6). However, reproducibility of the results was poor and a more reliable method was sought. The procedure finally adopted was based upon the finding of CRANE[10] that Norit A adsorbs most organic phosphorus compounds from acid solution, leaving inorganic orthophosphate and polyphosphate behind. In practice, 50–100 mg washed Norit A (the exact amount was not critical) were added to 6 ml of the PCA extract. After 10 min the suspension was centrifuged and the acid-labile phosphorus in the supernate was taken to represent the soluble polyphosphate. To justify this procedure it was shown that:

Virtually all (80–100 %) the phosphorus remaining after charcoal treatment was acid labile. Nucleotides, which are the major non-polyphosphate, acid-labile phosphorus compounds, were completely removed.

In experiments using ^{32}P, the acid-labile phosphate remaining after charcoal treatment was completely precipitable with Ba^{++} at pH 4.5.

It should nevertheless be pointed out that this method may give high values in cases where large amounts of non-nucleotide, acid-labile phosphorus compounds are present.

The ^{32}P content of the soluble polyphosphate was determined by precipitation with Ba^{++}. To 4 ml of the supernate from the charcoal treatment, 3.5 mg of carrier sodium polyphosphate (average chain length 50) were added, and the polyphosphate was precipitated as described above.

Lipid extract

Total phosphorus and total ^{32}P were determined as described above.

Hot PCA extract

Total phosphorus and total ^{32}P were determined as described above. Inorganic polyphosphate ("insoluble polyphosphate") was determined by adsorbing the nucleic acid on Norit A (about 200 mg Norit A/5 ml extract). The residual phosphorus, all of which was acid-labile, was taken to be inorganic polyphosphate. Total nucleic acids were estimated by difference, all the phosphorus adsorbed on charcoal being considered to be nucleic acid phosphorus. Frequently the extinction at 260 mμ was also determined and in a few cases the nucleic acid was eluted from the charcoal with alcoholic ammonia.

Residue

The residue from the hot PCA extract was analyzed for total phosphorus and total ^{32}P.

Chemicals

Reagent-grade chemicals were used whenever possible. Growth factors and antagonists were purchased from Nutritional Biochemicals Company or from the Sigma Chemical Company. ^{32}P was obtained from Oak Ridge National Laboratories. Norit A, purchased from Braun Chemical Company, was washed with dilute acid and with water before use.

Samples of inorganic polyphosphate were a gift from Dr. C. F. CALLIS of the Monsanto Chemical Company, and 2-thiazolealanine was a gift from Dr. R. G. JONES of the Lilly Research Laboratories. It is a pleasure to acknowledge their generosity.

RESULTS

Accumulation of acid-labile phosphate by Neurospora *strains*

In order to determine whether the accumulation of inorganic polyphosphate is a general property of *Neurospora* mutants or is correlated with specific metabolic blocks, a survey of mutants was undertaken. In all, 4 wild type and 49 mutant strains representing 40 different loci[11] were analyzed. The molds were grown in stationary cultures supplemented with various amounts of the required growth factor; the pads obtained after 4 days were analyzed for acid-labile phosphate extractable with cold PCA. Most *Neurospora* mutants were found to accumulate acid-labile phosphate when their growth was limited by the supply of available growth factor, but the amounts accumulated varied widely. Some of the experimental results are shown in Table I. No obvious correlation with specific metabolic blocks could be discerned.

From the work of HOULAHAN AND MITCHELL[2] it was already known that inorganic polyphosphate was the major constituent of the acid-soluble, acid-labile

TABLE I

ACID-LABILE PHOSPHATE IN PCA EXTRACT OF VARIOUS *Neurospora* STRAINS[11]

Locus	*Strain number*	*Supplement, mg/20 ml*		*Dry wt. of mycelium mg/20 ml*	*Acid-labile phosphate, mg/g mycelium*
Tryp-2	75001	Tryptophan,	2	100	1.9
			0.02	38	3.8
Tryp-4	C-151	Tryptophan,	2	25	1.4
			0.25	9	8.9
Nt	C-86	Tryptophan,	2	55	6.0
			0.15	13	14.2
Nt	39401a i	Tryptophan,	2	60	2.7
			0.25	30	12.7
Nic-1	3416	Niacin,	0.1	86	2.2
			0.005	52	7.9
Nic-1	44801	Niacin,	0.1	95	2.3
			0.005	60	4.1
His-1	C-84	Histidine,	2	77	5.6
			0.25	20	12.5
His-2	C-94	Histidine,	2	80	3.5
			0.25	15	10.5
His-3	C-140	Histidine,	2	73	5.4
			0.5	10	12.0
His-4	C-141	Histidine,	2	83	4.4
			0.5	13	8.5
Leu-1	33757	Leucine,	2	45	4.0
			0.25	11	5.9
Met-1	38706	Methionine,	2	92	4.8
			0.05	15	10.8
Met-5	9666	Methionine,	2	80	2.7
			0.1	12	12.5
pdx-p	39106	Pyridoxamine,	$12 \cdot 10^{-3}$	96	3.2
			$0.25 \cdot 10^{-3}$	35	5.3
pdx-q	35405	Pyridoxamine,	$12 \cdot 10^{-3}$	91	2.1
			$0.25 \cdot 10^{-3}$	41	4.3
Wild	25a	Biotin	$8 \cdot 10^{-5}$	80	2.0
			$1.6 \cdot 10^{-7}$	46	1.7
Wild	Em5297a	Biotin	$8 \cdot 10^{-5}$	94	2.5
			$1.6 \cdot 10^{-7}$	17	1.4

phosphorus fraction. For more specific identification of the material, cultures of the wild strain, 25a, and of 39401a (on limiting tryptophan) were grown on a large scale. Inorganic polyphosphate was isolated by precipitation with Ba^{++} at pH 4.5 as described in the experimental section. The washed barium salt was converted to the free acid by stirring with Amberlite resin IR-120 (see ref. 12); the latter was then analyzed by titration[13] and (after neutralization) by paper chromatography[14]. Both samples had an average chain length of about 20 by titration. Polyphosphates of chain length less than 7 were practically absent, in agreement with the results of MULLER AND EBEL[15] in other fungi.

Phosphorus compounds in shaken culture

To study the accumulation of polyphosphate as a function of time, shaken cultures of *Neurospora* were employed.

Wild type, 25a: As is the case in other micro-organisms, two types of inorganic polyphosphate occurred in *Neurospora*. One was extractable with cold PCA ("soluble polyphosphate") while the other was insoluble in cold PCA but was extracted by hot PCA ("insoluble polyphosphate"). The levels* of these and other phosphorus compounds at various times in a growing culture of the wild strain, 25a, are shown in Fig. 1. This pattern, remarkable only for the absence of dramatic changes, was quite typical of growing cultures of *Neurospora*, whether wild or mutant growing in the presence of excess of growth factor.

Histidineless, C-84: The histidine-requiring strain C-84 accumulates inorganic polyphosphate when grown on a limiting supply of histidine. To study the effect of histidine deprivation upon the levels of phosphorus compounds in C-84, a culture of C-84 was grown for 16 h in medium supplemented with 100 μg/ml histidine·HCl. The mycelium was then harvested and divided into two portions. One was resuspended in medium supplemented with histidine (flask A) while the other was placed in medium devoid of histidine (flask B). The subsequent behavior of these two cultures with respect to growth and phosphorus metabolism is shown in Fig. 2.

The culture in flask A continued to grow at a decreasing rate and exhibited no striking changes in the levels of the various phosphorus fractions. The starving culture in flask B grew normally for a time but growth ceased abruptly after 6 h. Coincident with the cessation of growth, there was a sharp rise in the level of soluble polyphosphate and a marked drop in the RNA content. The other phosphorus fractions, including the insoluble polyphosphate, were unaffected except for a slow rise in the level of inorganic orthophosphate.

The process of polyphosphate accumulation could be stopped at any time by addition of histidine to the starving culture. Thus, when histidine was added to a culture of C-84 which had been starved for 24 h, growth and RNA synthesis resumed while the accumulation of polyphosphate ceased. The polyphosphate content of the mycelium decreased, primarily due to admixture with newly synthesized mycelium of low polyphosphate content (Fig. 3). In experiments using ^{32}P, no extensive degradation of the accumulated polyphosphate was detected under these conditions. However, the polyphosphate was subject to degradation when no exogenous phosphate was supplied.

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* Throughout this paper, "level" refers to the amount/g dry weight.

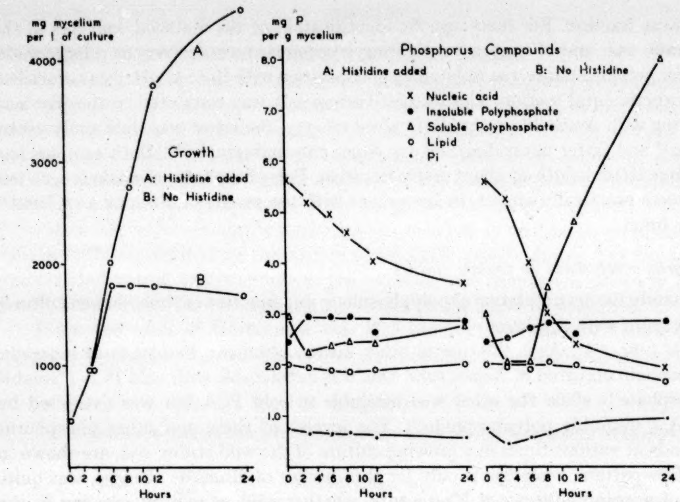

Fig. 2. Effect of histidine starvation on growth and phosphorus compounds in C-84. At 0 h, mycelium of C-84 was resuspended in fresh medium and divided into two portions. Flask A was supplemented with 100 μg/ml histidine while flask B received no histidine.

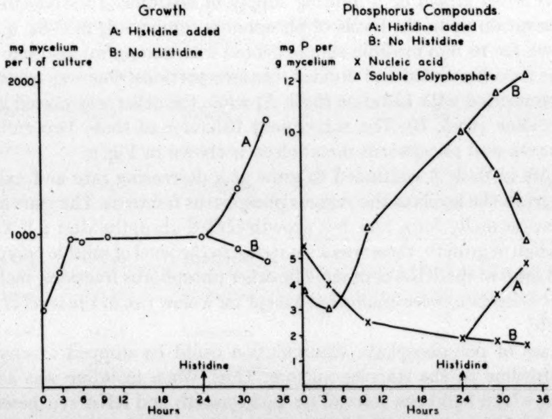

Fig. 3. Effect of histidine addition on growth and phosphorus compounds in C-84 starved of histidine. At 0 h, mycelium of C-84 was suspended in medium devoid of histidine. At 24 h, this culture was divided into two portions; histidine, 100 μg/ml, was added to flask A, while flask B received no histidine.

Results similar to those presented above were also obtained with other nutritional mutants. In general, polyphosphate accumulation in shaken cultures was less pronounced than in stationary ones. However, strains 38706 (methionineless) and 39401-RIA (nt-type, requiring either tryptophan or niacin), accumulated significant amounts of polyphosphate and showed the same reciprocal relationship of RNA and polyphosphate levels.

Growth inhibition and polyphosphate accumulation

The finding that polyphosphate accumulation was associated with a condition that limited growth, prompted numerous experiments on the effect of growth inhibition on polyphosphate metabolism. Most of these were performed with the wild strain 25a and with the tryptophan-requiring 39401-RIA, and were designed as described in the previous section. Inhibition of growth by depriving the cultures of nitrogen, sulfur, biotin or divalent metal ions or by the metabolic inhibitors *p*-fluorophenylalanine, azaserine, 5-methyltryptophan or ethionine failed to induce polyphosphate accumulation. These findings support the conclusion reached by HOULAHAN AND MITCHELL[2] that while the inhibition of growth may be a factor in polyphosphate accumulation it is not, in itself, sufficient to induce it.

A relationship between RNA and soluble polyphosphate

The results discussed in a previous section had hinted at a possible reciprocal relationship between RNA and polyphosphate. This matter was consequently explored by the use of ^{32}P.

Incorporation of ^{32}P into C-84 in presence and absence of histidine: A culture of C-84 which had been grown for 17 h in medium supplemented with 100 μg/ml histidine was harvested and divided into two equal portions. One was resuspended in medium supplemented with histidine (flask A) while the other (flask B) was placed in medium lacking histidine. When growth in flask B had ceased, ^{32}P was added to both cultures and its incorporation into various compounds was followed.

Some of the results are shown in Fig. 4. On a volumetric basis, the two cultures synthesized approximately equal amounts of soluble polyphosphate—culture A because it continued to grow and culture B because polyphosphate accumulated after growth had ceased. However, the incorporation of ^{32}P into the soluble polyphosphate was far greater in flask A than in flask B. It is clear that the bulk of the soluble polyphosphate synthesized by the starved mycelium was derived, not from exogenous phosphate, but from unlabeled, endogenous precursors.

^{32}P conservation in histidine-starved C-84: The nature of the endogenous source of phosphorus for polyphosphate synthesis became clear when the reverse experiment was performed. Mycelium of C-84 was grown in ^{32}P-labeled medium supplemented with 100 μg/ml histidine for 17 h, harvested, resuspended in fresh, unlabeled medium and divided into two portions. Flask A was supplemented with histidine while flask B received none. The redistribution of the ^{32}P was followed and is shown in Fig. 5.

In the growing culture (flask A), the nucleic acids retained virtually all of the ^{32}P incorporated into them. This behavior is typical of most growing micro-organisms[16, 17]. In flask B, growth ceased after 6 h and rapid degradation of nucleic acid set in. The bases were apparently excreted into the medium, but the phosphorus was retained within the mycelium and converted to soluble polyphosphate. The ^{32}P content of the insoluble polyphosphate fraction did not increase, but a slow rise in the inorganic orthophosphate fraction was generally seen. The behavior of other fractions, not shown in Fig. 5, was the same in both cultures. Nucleic acids thus constituted a major source of phosphorus for polyphosphate accumulation, though it should be noted that

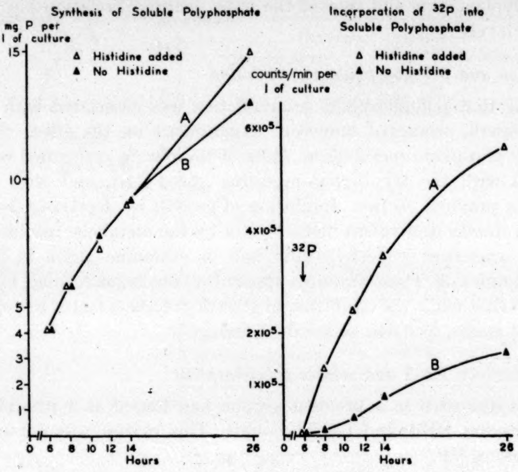

Fig. 4. Incorporation of ^{32}P into the soluble polyphosphate of C-84 in the presence and absence of histidine. At 0 h, mycelium of C-84 was resuspended in fresh medium and divided into two portions. Flask A was supplemented with histidine while flask B was not. ^{32}P was added to both at 6 h. Ordinates refer to amounts of phosphorus or ^{32}P in polyphosphate per unit volume of culture.

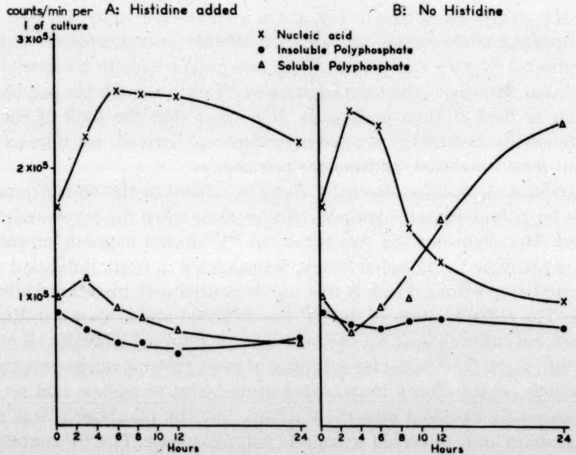

Fig. 5. Retention of ^{32}P in phosphorus compounds of C-84 in presence and absence of histidine. At 0 h, ^{32}P-labeled mycelium of C-84 was resuspended in fresh medium and divided into two portions. Flask A received histidine while B did not. To facilitate quantitative comparisons, the data on growth and phosphorus fractions pertaining to this experiment were plotted in Fig. 2. Ordinates as in Fig. 4.

the amount of polyphosphate synthesized was greater than that of phosphorus released from the nucleic acids.

Since DNA is a relatively minor component of the nucleic acids of *Neurospora*, amounting to less than 10 % of the total[18], it is clear that RNA must be undergoing degradation. In fact, no net degradation of DNA has yet been observed as measured by BURTON's method[19].

Polyphosphate accumulation as a consequence of RNA degradation

The finding that RNA degradation constitutes a major source of phosphorus for polyphosphate accumulation raises the question as to whether these two phenomena are related as cause and effect. To test this hypothesis a more detailed investigation of the correlation between RNA degradation and polyphosphate accumulation was undertaken.

Reversal of RNA degradation by readdition of histidine: When histidine was added to a culture of C-84 which had been starved of histidine, the degradation of RNA ceased. In fact, growth resumed and the accumulation of polyphosphate also ceased (see a previous section).

Failure of various starvation conditions and growth inhibitors to induce RNA degradation: In a previous section, a number of treatments were listed which inhibited growth but did not induce polyphosphate accumulation. In general, RNA degradation also failed to occur under those conditions*. The effect of nitrogen deprivation on C-84 illustrates this conclusion. When mycelium of C-84 was transferred to medium devoid of nitrogen, the synthesis of protein and of RNA ceased abruptly, and that of other phosphorus compounds soon followed suit. However, no significant loss of ^{32}P from RNA was observed. It is of interest that the mass of the mycelium continued to increase for some time, often doubling or tripling before "growth" ceased. This is presumably due to accumulation of "reserve materials" such as lipids or polysaccharides[20].

RNA degradation and polyphosphate accumulation in other Neurospora *mutants*: Studies on ^{32}P conservation, analogous to those described for C-84, were also carried out with a number of other mutants. In every case, RNA degradation was observed when mycelium of an auxotrophic strain was grown on fully supplemented medium and then transferred to minimal medium. However, the pattern of growth, RNA degradation and polyphosphate accumulation varied from strain to strain.

The methionineless strain, 38706, continued to grow very slowly in the absence of methionine. Polyphosphate accumulation was comparable to that in C-84 and transfer of ^{32}P from RNA to polyphosphate was demonstrated, but the stoichiometry was obscured by the continued synthesis of RNA.

A different pattern was found in the leucineless strain, 33757. When mycelium of this strain was transferred to minimal medium, degradation of nucleic acid set in immediately. The phosphorus was largely converted to soluble polyphosphate, resulting in limited accumulation of this substance (Fig. 6). Increase of the mycelial mass partly obscured this accumulation when the polyphosphate content was expressed as mg P/g mycelium (maximum value observed: 3.0 mg P/g).

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* Sulfur starvation did induce some RNA degradation in C-84, and there was a concomitant increase in the ^{32}P content of the soluble polyphosphate fraction. Net accumulation of polyphosphate was not observed, perhaps because the culture continued to grow slowly.

The behavior of a double mutant requiring both leucine and histidine was also studied. This strain was prepared by crossing C-84a and 33757A, using the latter as the conidial parent. The pattern of growth, RNA degradation and polyphosphate accumulation depended upon which of the two amino acids was withheld: when histidine alone was absent, it behaved like C-84 while in leucine deficiency it behaved like 33757. When neither amino acid was supplied, the pattern seen in leucine starvation was obtained.

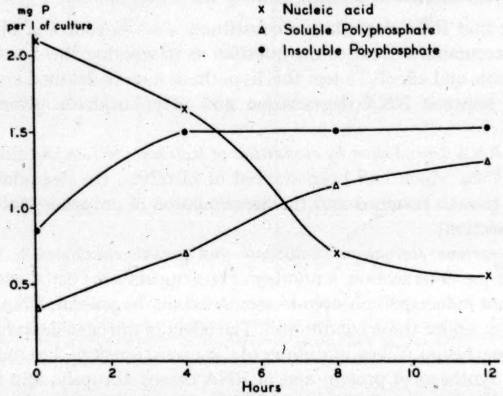

Fig. 6. Effect of leucine starvation on intracellular phosphorus compounds in 33757. Mycelium of 33757 was transferred to medium lacking leucine at 0 h. Ordinate as in Fig. 4.

Finally, the tryptophanless strain C-83, degraded RNA but accumulated practically no polyphosphate.

Induction of RNA degradation by sucrose starvation: As pointed out above, starvation of *Neurospora* with respect to most of the major nutrients did not induce RNA degradation. However, in all the strains tested, RNA degradation did ensue when the mycelium was suspended in medium lacking the carbon source, sucrose. It thus became possible to study the fate of RNA-phosphorus in non-growing cultures.

A culture of C-84 was grown in ^{32}P-labeled, supplemented medium for 17 h. The mycelium was then harvested and resuspended in unlabeled medium, containing histidine but lacking sucrose. No increase in mass occurred under these conditions—in fact, a small loss of weight was observed. As shown in Fig. 7, considerable degradation of nucleic acid took place. The insoluble polyphosphate and the phospholipid also showed some degradation while orthophosphate and soluble polyphosphate accumulated.

These changes were due almost entirely to a redistribution of the intracellular phosphorus, and resulted in its retention within the mycelium. Generally, 80–90 % of the ^{32}P originally present in the mycelium was conserved. Also, parallel experiments, in which unlabeled mycelium was resuspended in labeled medium lacking sucrose showed that virtually no incorporation of phosphorus into the mycelium took place under these conditions. Moreover, the results of Fig. 7 could be duplicated by suspending the mycelium in buffer lacking both nitrogen and phosphorus as well as sucrose.

Parallel experiments were carried out with several other strains of *Neurospora*, both wild and mutant. RNA degradation could be demonstrated in all strains, but the amount of polyphosphate accumulated varied. Some strains behaved in a manner analogous to C-84: 38706 and 39401 RIA, and the wild types Em5297a and 1A fell into this category. Other strains, including C-83 and the wild type 25a, degraded RNA extensively yet synthesized little or no polyphosphate. Intermediate responses were also found.

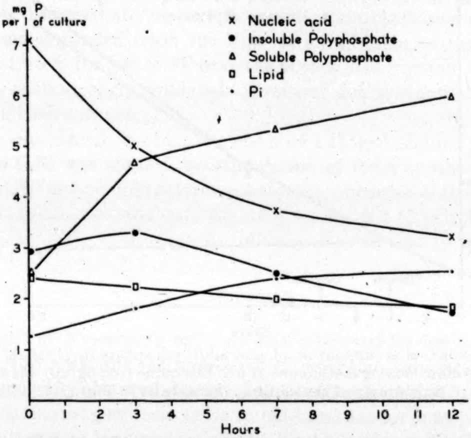

Fig. 7. Effect of sucrose starvation on intracellular phosphorus compounds in C-84. Mycelium of C-84 was resuspended in medium lacking sucrose at 0 h. Ordinate as in Fig. 4.

It is clear from these results that RNA degradation can be a sufficient cause of polyphosphate accumulation by both mutant and wild strains. The efficiency with which phosphorus released from RNA is converted to polyphosphate appears to vary with the strain and may be the resultant of a number of competing reactions.

Inhibition of polyphosphate accumulation and of RNA degradation

Amino acid analogs: In the course of the experiments discussed in a previous section it was discovered that certain amino acid analogs inhibited the accumulation to polyphosphate. In general, an analog of the amino acid required by the mutant was most effective. Thus polyphosphate accumulation in strain 39401-RIA was inhibited by 5-methyl tryptophan (400 μg/ml) which had little effect on C-84 or 38706. Conversely, polyphosphate accumulation in 38706 was strongly inhibited by ethionine (100 μg/ml) while C-84 and 39401-RIA were scarcely affected. No inhibition of RNA degradation was observed.

The degree of inhibition of polyphosphate accumulation by a given concentration of analog was strongly dependent upon the time of addition of the inhibitor. This is illustrated in Fig. 8, which describes the effect of ethionine, added at various times to a culture of 38706 (methionineless) starved for methionine. It is perhaps significant that early addition of ethionine also caused a marked loss of mycelial mass.

The mechanism of this effect remains to be clarified. However, the above results seem to suggest that protein synthesis must be allowed to proceed during the early stages of polyphosphate accumulation. This suggestion receives support from the finding that when mycelium of C-84, deprived of histidine, was transferred at various times to fresh medium devoid of nitrogen, a similar effect was observed. Nitrogen

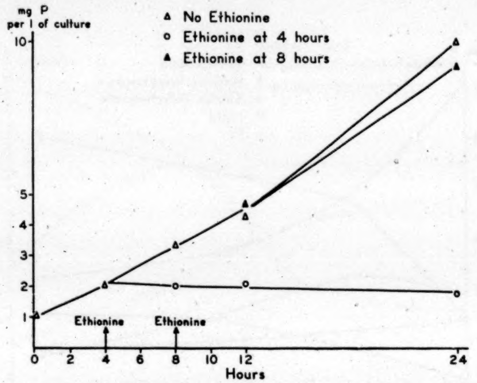

Fig. 8. Effect of ethionine on accumulation of acid-labile phosphate in 38706. Mycelium of 38706 was transferred to medium lacking methionine at 0 h. Ethionine (100 μg/ml) was added at 4 and 8 h to aliquots of the culture. Ordinate as in Fig. 4.

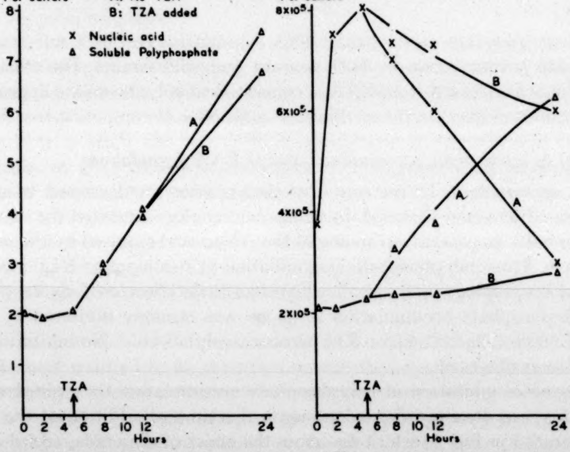

Fig. 9. Effect of TZA on polyphosphate synthesis and ^{32}P conservation in C-84. Mycelium of C-84 was transferred to medium lacking histidine at 0 h (A). At 5 h, 200 μg/ml of TZA were added to an aliquot (B) of the culture. Ordinates as in Fig. 4.

deprivation inhibited the onset of polyphosphate accumulation but had little effect at later times. It seems likely that the role of protein synthesis is an indirect one, perhaps involving leakiness of the cell as loss of intracellular constituents was often observed.

2-thiazolealanine: This analog of histidine[21] proved to have the disturbing property of inhibiting RNA degradation in C-84 with little effect on polyphosphate accumulation. The results of an experiment in which 200 μg/ml 2-thiazolealanine were added to a culture of C-84 deprived of histidine are shown in Fig. 9. As was the case with the analogs discussed above, the efficacy of 2-thiazolealanine as an inhibitor of RNA degradation depended upon the time of its addition to the culture. In the experiment illustrated, the loss of ^{32}P from the nucleic acid fraction, and its transfer to polyphosphate, were strongly inhibited. However, net synthesis of polyphosphate was the same in both cultures.

In a single experiment in which the effect of 2-thiazolealanine on the incorporation of ^{32}P into C-84 was studied, no stimulation of RNA synthesis was observed; incorporation of ^{32}P into soluble polyphosphate was, of course, enhanced. The amount of available thiazolealanine was unfortunately too limited to permit more extensive investigation.

DISCUSSION

When the growth of *Neurospora* mutants was restricted by limiting the supply of their required growth factors, accumulation of inorganic polyphosphate within the mycelium was generally observed. The level of polyphosphate that accumulated varied from strain to strain, ranging from twice to ten times the amount present in the wild type. We must therefore inquire how genetic blocks in the synthesis of a wide range of cellular constituents bring about the accumulation of this substance. The obvious suggestion that limitation of growth per se is the condition that induces polyphosphate accumulation, seems to be ruled out by the experiments presented here. This conclusion reinforces that already reached by HOULAHAN AND MITCHELL[2]. At the same time, the very generality of the phenomenon argues against the view that polyphosphate accumulation is a direct consequence of specific metabolic blocks. It appears most plausible to suppose that the various nutritional deficiencies act by inducing a common disturbance of phosphorus metabolism.

A major disturbance of phosphorus metabolism, which was observed in all the mutants studied when the supply of growth factor was exhausted, was the degradation of intracellular RNA. Acid-soluble polyphosphate accumulated in many mutants at the same time. The phosphorus released from RNA was thereby retained within the mycelium and served as a major source of phosphorus for polyphosphate synthesis. As polyphosphate could also be synthesized from exogenous phosphate, without significant RNA turnover, (*e.g.*, during normal growth) it is reasonable to postulate a pool, or pools, of intermediates common to both pathways. The nature of these intermediates is as yet unknown, but ATP in particular is a likely candidate[22–24]. These interrelations are schematically represented in Fig. 10.

Since degradation of RNA constituted a major source of phosphorus for polyphosphate accumulation, the question arises to what extent RNA degradation may be regarded as the immediate cause of this phenomenon. In terms of the histidine-requiring mutant, C-84, this formulation of the problem implies the following hypothesis: Histidine deprivation induces the breakdown of RNA (reaction 1). Phosphorus liberated from RNA would then flow into the pool of precursors postulated above and polyphosphate accumulation would follow by virtue of the increased production of precursors.

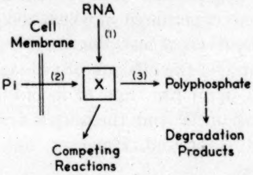

Fig. 10. Metabolic interrelations between RNA and soluble polyphosphate.

This hypothesis offers a ready explanation for the correlation of polyphosphate accumulation with RNA degradation. Strong evidence for the validity of the proposed chain of reactions comes especially from the finding that sucrose starvation induced degradation of RNA and that polyphosphate accumulated under these conditions. This was the case, not only in C-84 and other mutants, but even in many wild strains. Thus, release of phosphorus from RNA appears to be a sufficient cause for polyphosphate accumulation. The observation that the amount of polyphosphate accumulated in histidine starvation exceeded that of RNA degraded can be accounted for, at least in part, by the simultaneous synthesis and breakdown of RNA which were found under these conditions.

While RNA degradation is thus most probably an important factor in polyphosphate accumulation it nevertheless does not seem to be the whole story. This is brought out clearly by the observation that 2-thiazolealanine inhibited RNA degradation in C-84 without affecting polyphosphate accumulation. Whatever the explanation of this finding may be, it implies that other processes, besides RNA degradation, may bring about polyphosphate accumulation. For example, it is conceivable that histidine deprivation in C-84 not only induces RNA degradation but also affects other steps leading to polyphosphate synthesis (reaction 2 or 3). If this were the case, RNA phosphorus entering the precursor pool might be used preferentially for polyphosphate synthesis, but would not be an obligatory precursor: when RNA degradation is artificially inhibited, phosphate would be taken up from the medium. Experiments now in progress on the nature of the precursor, X, may clarify this point.

The complex metabolic control of polyphosphate accumulation suggested by the experiments with strain C-84 provides a reasonable basis for speculation on the wide range of polyphosphate levels attained by various other mutants. Some strains, such as the methionine-requiring 38706, appear to be analogous to C-84 and accumulate large amounts of polyphosphate. Whether this polyphosphate is entirely derived from RNA turnover, or arises in part from other processes, remains to be determined. In other mutants, such as the leucineless strain 33757, polyphosphate accumulation is more limited and can be completely accounted for by the phosphorus liberated from RNA. In this connection, the results with the double mutant that requires both histidine and leucine are of interest. Apparently the nature of the metabolic block, by influencing the pattern of growth, and RNA degradation, is one of the parameters that determine the amount of polyphosphate accumulated. Finally, in mutants such as the tryptophanless strain C-83, which fail to accumulate polyphosphate, one may expect to find reactions that compete for RNA-phosphorus, resulting in the ultimate loss of phosphorus from the mycelium. Leakiness of the cell membrane and degradation of polyphosphate may also limit, or prevent, polyphosphate accumulation.

It remains to consider the accumulation of polyphosphate by other microbial species in the light of the present findings in *Neurospora*. Polyphosphate accumulation is frequently encountered in cultures of yeast, bacteria and algae under conditions unfavorable to growth (see *e.g.*, ref. 7, 24, 25) and is commonly interpreted as resulting from excessive production of ATP which is not utilized in synthetic reactions[23, 26, 27]. A reciprocal relationship between the synthesis of RNA and of polyphosphate has also been postulated[28, 29]. The results presented here suggest that degradative reactions, particularly the degradation of RNA[30–32], may have to be taken into account in some of these cases.

A unique feature of polyphosphate accumulation in *Neurospora* is that it is confined to the acid-soluble fraction. This observation must be added to the growing evidence supporting the view that the acid-soluble and -insoluble polyphosphates constitute physiologically distinct entities[33, 34].

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Short Communications

Extraction d'un acide desoxyribonucleique transformant par le dodecyl sulfate de sodium et le phenol

Il est difficile de préparer à partir de tissus ou de bactéries infectés par des virus de l'ADN ne contenant pas de virus résiduel. Dans le cas des ARN biologiquement actifs, préparés par la méthode de Gierer et Schramm[1] à partir de matériaux contenant des virus divers en quantité variable, on ne détecte pas de virus infectieux.

En préparant l'ARN par cette méthode à partir de tissus, nous avons observé que l'extrait final contenait parfois des quantités appréciables d'ADN. Nous avons cherché à augmenter le plus possible le rendement en ADN. Dans ce but, nous avons essayé d'ajouter aux préparations du dodecyl sulfate de sodium avant le traitement au phénol. Ceci pour les raisons suivantes: Kirby[2] a montré que l'addition de certains anions avant le traitement par le phénol permet à l'ADN de rester avec l'ARN dans la phase aqueuse. Sreensivasaya et Pirie[3] ont utilisé le SDS pour dissocier la nucléoprotéine de la mosaïque du tabac et Marko et Butler[4] ont développé une méthode de préparation des acides nucléiques avec ce détergent.

Pour avoir un contrôle des qualités biologiques de l'ADN ainsi préparé nous avons utilisé la transformation du pneumocoque R36A vers la résistance à la streptomycine.

La souche de pneumocoque est cultivée en milieu 1 indiqué par Ephrussi-Taylor[5]. En fin de croissance, la culture est divisée en deux parties. Un lot sert à la préparation des acides nucléiques selon la méthode de McCarthy et Avery[6]: on lyse les cellules par le désoxycholate en présence de citrate, on déprotéinise selon la technique de Sevag[7] puis on précipite les acides nucléiques par l'éthanol.

L'autre partie est centrifugée. Les bactéries sont lavées et diluées dans un tampon phosphate 0.02 *M*–ethylènediaminetétraacétate 0.01 *M* pH 7.2, de manière à avoir $5 \cdot 10^8$ à $2 \cdot 10^9$ bactéries/ml (le rendement en ADN diminue si la concentration bactérienne est plus élevée). On ajoute du SDS (solution à 20 % dans du tampon phosphate 0.2 *M*, pH 7.3) pour avoir une concentration finale de 0.4 %. On agite 3–5 min dans l'appareil de Mickle[8]; on ajoute un volume égal de phénol saturé d'eau (phénol BDH Analar). On agite 5 min. Après centrifugation, on prélève la phase aqueuse sur laquelle on répète le traitement au moins deux fois, parfois plus, suivant le degré de purification désiré.

La phase aqueuse est finalement lavée à l'éther (5–6 fois).

Les acides nucléiques sont précipités rapidement par 2 vol. d'éthanol froid, en présence de 2 % d'acétate de sodium. Le précipité est tout de suite séparé par centrifugation pour éviter d'entraîner du SDS (ce dernier précipitant dans l'alcool plus lentement que les acides nucléiques).

Abbréviations: ADN, acide déoxyribonucléique; ARN, acide ribonucléique; SDS, dodécyl sulfate de sodium.

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Les précipités sont lavés 4 fois par un mélange éthanol (2 vol.) NaCl 0.14 *M* (1 vol.) puis dissous dans du NaCl 0.14 *M*–glycocolle 0.01 *M*.

L'activité transformante a été testée sur des bactéries compétentes congelées suivant la technique indiquée par FOX ET HOTCHKISS[9].

L'ADN a été dosé selon la méthode de KECK[10] et de DISCHE[11] et les acides nucléiques totaux déterminés par l'absorption à 258 mμ; les protéines ont été déterminées par la méthode de LOWRY[12].

Le Tableau I donne la moyenne des résultats obtenus sur 6 préparations.

TABLEAU I

	Extraction par SDS et phénol	*Extraction selon* MC CARTHY
Rendement en ADN rapporté à une quantité de bactéries correspondant à 10 mg de poids sec (μg)	250	200
Pourcentage d'ADN par rapport aux acides nucléiques totaux	12,3	19
Protéine en pour cent d'acide nucléique total après 3 déprotéinisations	< 1	2.5
Viscosité réduite $\left(\frac{\eta sp}{c}\right)$	3000	5790
Spectre u.v.		
$\frac{\varepsilon\text{max } (258\ \text{m}\mu)}{\varepsilon\text{min } (230\ \text{m}\mu)}$	2.37	2.02
$\frac{\varepsilon 258\ \text{m}\mu}{\varepsilon 280\ \text{m}\mu}$	2.18	2.02
Nombre de transformations par μg d'ADN (par extrapolation de la partie linéaire)	$6.2 \cdot 10^5$	$4.5 \cdot 10^5$

Nous avons ajouté aux bactéries avant l'extraction des quantités déterminées de virus afin de vérifier leur élimination.

Le Tableau II donne les résultats pour 3 phages et 1 virus animal.

TABLEAU II

	Nombre de bactériophages survivants sur 10^{10} ajoutés initialement
T_{2r} après 3 extractions au phénol	0
Après 6 Sevag	10^5
T_3 après 3 extractions au phénol	0
Phage 167 après 3 extractions au phénol	10
Après 4 extractions au phénol	0
Après 3 Sevag	$2 \cdot 10^8$
Après 5 Sevag	$5 \cdot 10^5$
Avec le virus de l'encéphalomyocardite, pour $5 \cdot 10^8$ PFU au départ, il reste:	
Après 3 extractions au phénol	0
Après 3 Sevag	$5 \cdot 10^5$

Nous avons essayé différents milieux pour faire l'extraction: NaCl 0.14 *M*, NaCl 1 *M*, tampon citrate 0.1 *M*, milieu de EARLE et nous avons aussi suivi l'effet de la température (4°, 22°, 37°). Nous n'avons pas constaté de différences significatives dans le produit final. Le résultat le plus constant a été obtenu en opérant à 4°. Le milieu de EARLE[13] a parfois permis d'obtenir un rendement plus élevé.

L'activité transformante spécifique est en moyenne supérieure de 33% à celle des extraits préparés par la méthode MCCARTHY. La viscosité, environ deux fois plus faible, indique un poids moléculaire plus bas. Nous n'avons pas assez de données pour discuter ici une relation éventuelle entre l'augmentation de l'activité spécifique et la faible viscosité.

La marqueur Sm, qui n'occupe qu'une partie de la molécule[14], n'est peut-être pas un critère suffisant pour juger de l'intégrité de l'ADN étudié. La même méthode a permis néanmoins, de préparer, à partir de bactéries infectées, un ADN capable de reproduire des phages[15].

La technique est rapide et permet facilement de manipuler de petits échantillons. Elle permet d'obtenir, avec un bon rendement, de l'ADN de toutes sortes de tissus normaux et tumoraux. Toutefois, il est nécessaire de choisir, dans chaque cas, la dilution du matériel initial la mieux adaptée au tissue utilisé car une trop forte concentration empêche la séparation de l'ADN.

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The lability of rat-liver microsomes and ribonucleoprotein particles

That microsomes lose their ability to incorporate amino acids when kept at room temperature for a short period or at 4° overnight has been repeatedly observed. SACHS has noted that S-protein, an RNA-free fraction of cell sap, will partially stabilize microsomes[1]. To our knowledge, however, no kinetic study of the decay process has been carried out. In this paper we report the results of such studies on rat-liver microsomes and on active ribonucleoprotein particles obtained from the same source.

Once-washed microsomes were prepared from 200-g Wistar rats by a procedure based on the method of KELLER AND ZAMECNIK[2], and the modification of SACHS[1]. Sucrose (0.25 M) was used both for the homogenization step and for washing the microsomes. Ribonucleoprotein particles were prepared according to the procedure of RENDI AND HULTIN[3]. This method employs the detergents Lubrol WX (a polymer of ethylene oxide and a long-chain fatty alcohol, Arnold Hoffman and Co., Providence, R.I.) and sodium deoxycholate (Matheson, Coleman and Bell, Cincinnati, Ohio) to liberate particles from endoplasmic reticulum. The partially clarified mitochondrial supernatant obtained is layered onto a medium of higher density and centrifuged at 105,000 × g. This process separates the dense particles from the cell sap and dispersed reticulum.

The decay of microsomes or ribonucleoprotein particles was followed by pre-incubating suspensions in the desired medium and, after a variable period, adding the balance of salts, cell sap, radioactive leucine, and adenosine triphosphate-generating system necessary for incorporation. Incubation of the mixture (microsomes, 1 ml; ribonucleoprotein particles, 0.5 ml) was carried out at the same temperature as the pre-incubation for a standard period (20 or 30 min). The incorporation of radioactive leucine into protein was estimated in the usual way. Since in a given experiment all tubes contained the same amount of protein, no correction for self-absorption was necessary. The observed incorporations, were, however, multiplied by a factor such that the incorporation, i, for zero pre-incubation, $t = 0$, was equal to 100. This adjustment made it easier to compare the results of different experiments.

The results of a typical microsome-decay experiment at 37° are shown in Fig. 1a. The semi-log plots of such experiments show an initial curved region of fast decay which eventually approaches a straight line, *i.e.*, the decay process approaches the time course of a first-order reaction–simple exponential decay. Both the extent of the curved region and the slope of the linear region vary somewhat from experiment to experiment. The rate of decay is markedly affected by temperature; thus at 22° the incorporation was reduced by 16 % after 30 min as compared with 80 % at 37°. Similar decay curves were observed for ribonucleoprotein particles (7 mg protein/ml) in Tris buffer (0.035 M), Tris–KCl (0.05 M–0.2 M) and $MgCl_2$–KCl (0.01 M–0.2 M). The slope of the linear portion in these experiments was about half that found for microsomes.

If only a single species of labile particle capable of incorporation were present, the number of such particles decreased during pre-incubation according to first-order kinetics, and the observed incorporations were proportional to the number of active particles present at the start of the incubation, then a straight line would have been observed throughout the pre-incubation period. The form of the experimental curve

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Abbreviations: RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

suggests that particles decaying at appreciably different rates are present. In Fig. 1a, selected to document fully the curved region, it is shown that the experimental points may be adequately represented by the sum of two first-order decay terms. The presence of two independently decaying species with half lives differing by a factor of 10 (2.9 min and 30 min) is therefore postulated. In agreement with this, the form of the curves for the time course of incorporation into microsomes at 37° may be accounted for by assuming linear incorporation into two species of decaying particles.

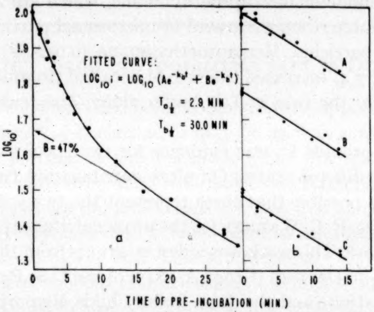

Fig. 1. (a) Microsome decay at 37°: microsome protein concentration, 1.5 mg/ml. (b) Microsome decay at 29° as a function of microsome concentration; microsome protein concentration A:B:C = 4:2:1, curve A, 5.2 mg/ml. The pre-incubation mixtures (0.8 ml) contained all the incubation constituents less cell sap, radioactive leucine, and 20 μmoles sucrose. Incubation mixture (1.0 ml): creatine phosphate (K^+), 20 μmoles; adenosine triphosphate (K^+), 1 μmole; creatine kinase, 140 μg(guanosine triphosphate (Na^+), 0.25 μmole; $MgCl_2$, 5 μmoles; KCl, 80 μmoles; glutathione (K^+), 5 μmoles; Tris–HCl buffer, pH 7.4, 30 μmoles; sucrose, 100 μmoles; radioactive, L-leucine, 0.1 μmole (200,000 counts/min; obtained from Nuclear-Chicago Corp.); and cell sap, 0.8 mg. All solutions were adjusted to pH 7.4 before mixing.

Measurements of the rate of sedimentation, at 20°, of lubrol–deoxycholate ribonucleoprotein particles were made with a model E Spinco ultracentrifuge fitted with Schlieren optics. Two major components with sedimentation coefficients of the order of 50 and 70 were observed in the following media (pH 7.8, protein concentration 5 mg/ml): Tris–lubrol (0.02 M–0.2 %), Tris–lubrol–KCl (0.02 M–0.2 %–0.2 M), Tris–$MgCl_2$ (0.02 M–0.0001 and 0.002 M). These two species may correspond to the components C (63S) and B (83S) found by HAMILTON AND PETERMAN[4] in rat-liver microsomes (sedimentation coefficients corrected to zero protein concentration). It is possible that these two kinds of particles are both capable of incorporation and that they decay at different rates.

The rate of microsome decay was found to be independent of microsome concentration over a 4-fold range of dilution (Fig. 1b). For this experiment, a temperature at which it was easily possible to follow the initial rate of decay was selected; 29° instead of 37°. Were the decay due to an enzyme released from the microsomes into the suspension medium, then the more dilute solutions would be expected to show a reduced rate of decay. To account for the observed results the enzyme, *e.g.* ribonuclease, would have to be attached to the ribonucleoprotein particle in such a manner that the enzyme could attack ribonucleic acid associated with its own particle but could not attack that of other particles. It seems more probable that the decay of the particles should be ascribed to denaturation[5,6] or to spontaneous chemical changes. In this connection it may be mentioned that incubation, in 0.5 *M* NaCl, of ribonucleoprotein particles at 27° in pH-7 buffer for 24 h did not release measurable amounts of acid-soluble oligonucleotides (see ref. 7).

Thiols, *e.g.*, glutathione or mercaptoethylamine, which are required for transfer of amino acids from soluble ribonucleic acid to microsomal particles[8] markedly affect the stability of these particles. Mercaptoethylamine (0.012 *M*) in the presence of Tris buffer (0.05 *M*, pH 7.4) increased the rate of decay of ribonucleoprotein particles at 37° about 3-fold over the rate in Tris buffer alone. The reason for this effect is unknown.

The above results provide kinetic evidence for two species of ribonucleoprotein particle which decay at different rates. On ultracentrifugation two main components were observed, and it is possible that these represent the two species in question.

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Preliminary Notes

The 1680 cm^{-1} band of ribonucleic acid in D_2O solutions

In our previous paper[1], it has been pointed out that the appearance of the absorption band at about 1710 cm^{-1} of nucleic acids may be used as a criterion of the existence of the specific base pairing. It was considered that the absorptions in the 1700–1720 cm^{-1} region are caused by the C=O and C=N stretching motions of cytosine and adenine residues, where the π-electrons are localized at these bonds to a greater extent than in the free cytosine and adenine residues, and that such a localization of the π-electrons is caused by the base–base interactions.

From our infrared studies of nucleosides[2], evidence has been obtained that absorption due to the stretching vibrations of the deuterated base residues, corresponding to absorption in the 1700–1720 cm^{-1} region of the undeuterated base residues, appears in the 1670–1690 cm^{-1} region. Therefore, for the D_2O solutions of nucleic acids the appearance of absorption in the vicinity of 1680 cm^{-1} may be used as a criterion of the existence of the base pairings.

Some of the results of our measurements of the infrared spectra of sodium salt of ribonucleic acid, prepared by the phenol method from ribosomes of *Escherichia coli*, in D_2O solution are shown in Fig. 1. It can be seen that it has a strong absorption band in the 1680 cm^{-1} region, which is a little weakened in intensity upon heating at 100° for 10 min. When the sodium ribonucleate is treated with ribonuclease, the 1680 cm^{-1} absorption is much weakened.

Blout and Lenormant[3] found that high-polymer sodium deoxyribonucleate shows a strong band at 1680 cm^{-1} in D_2O solution, and that, when the solution is heated,

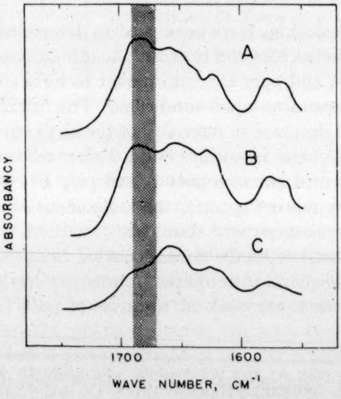

Fig. 1. Infrared-absorption curves of sodium ribonucleate from *E. coli* ribosomes in D_2O solution. A, native; B, heated to 100° for 10 min; C, treated with ribonuclease.

treated with deoxyribonuclease, or treated with alkali, its 1680 cm^{-1} band is much weakened. They also found that the low-polymer sodium deoxyribonucleate does not show the strong 1680 cm^{-1} band.

All these facts indicate, according to the interpretation based on the criterion suggested above, that the base paring takes place only in the high-polymer ribonucleic and deoxyribonucleic acids but not in the low-polymer nor in the denatured forms.

We have also observed 6-μ-region spectra of various nucleic acids and nucleoproteins in D_2O solution. The results of the observations and the interpretations on the basis of the criterion suggested here will be detailed elsewhere.

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Infrared spectra of the three-stranded helices formed by polyuridylic acid with polyadenylic acid and with tetraadenylic acid

Infrared spectra in D_2O solutions have been used to determine the tautomeric forms of nucleotides and polynucleotides and to study the interactions of polynucleotides in aqueous solution[1]. Poly A and poly U[*] can interact to form either a two-stranded or a three-stranded helix depending upon conditions[2]. The infrared spectra of the two-stranded helix[1] showed a decrease in intensity of the 1632 cm^{-1} band and frequency increase of this band and of the 1660 cm^{-1} band. This note reports the spectra of the three-stranded helices formed between poly A and poly U and between tetra A and poly U. For the necessary related spectra, the assignment of bands, and the experimental methods used, previous papers[1] should be consulted.

The most striking result with the three-stranded helix concerns the 1630 cm^{-1} band. As stated above, this band is decreased in intensity in the two-stranded helix[1]. It has disappeared as a separate peak in the case of poly (A + 2 U) and of tetra A + 2 poly U (Fig. 1).

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[*] The polyadenylic acid (poly A) and polyuridylic acid (poly U) were prepared with polynucleotide phosphorylase (M. GRUNBERG-MANAGO AND S. OCHOA, *J. Am. Chem. Soc.*, 77 (1955), 3165). The tetraadenylic acid (tetra A = pApApApA)[3] + 2 poly U solution was the generous gift of Dr. MARIE LIPSETT AND Dr. LEON HEPPEL[3].

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It has been shown that polynucleotide helices[3,4] dissociate reversibly upon heating, the melting temperature depending upon the polymers used and upon the kind and concentration of salt. By using heated solutions it has been possible to follow the thermal dissociation of these three-stranded helices (Fig. 1; Table I) by their infrared spectra. The hot solution of tetra A + 2 poly U shows a fully developed peak at 1625 cm^{-1} (Fig. 1E), indicating complete dissociation of the helix. As the solution slowly cools there is a gradual decrease in intensity of this peak until the original spectrum (Fig. 1D) is again observed at room temperature. In the case of poly (A + 2U) this spectral effect was much less pronounced (Fig. 1C) since the attainable temperature was not high enough to cause extensive dissociation. It has been proposed that a major factor contributing to the changes in the infrared spectra of polynucleotides upon helix formation is the decrease in dielectric constant as the water is forced away from the planar aromatic rings by the formation of the very tightly packed helix[5]. When a second strand of poly U is added, the solvent would be further excluded from the vicinity of the adenine rings. It is possible that this further decrease in dielectric constant around the adenine rings may contribute to the further decrease in intensity of the adenine absorption, though the effect of induced

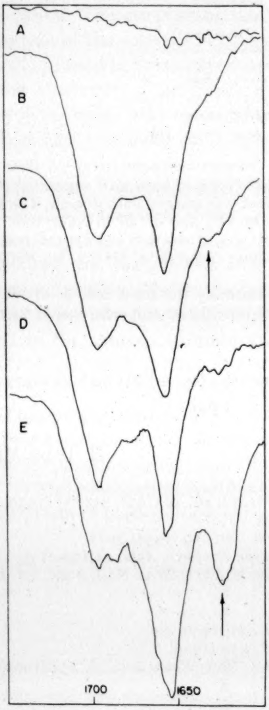

Fig. 1. Infrared absorption spectra in D_2O of polynucleotide solutions. Abscissa, frequency of absorption in cm^{-1}. Ordinate, % transmission on arbitrary scale. Conditions indicated in footnote to Table I. A, D_2O *vs.* D_2O to show compensation obtained. B, Poly A + 2 poly U at 30°. C, Poly A + 2 poly U at about 60°; the arrow at 1632 cm^{-1} indicates the slight increase in absorption. D, Tetra A + 2 poly U at 30°. E, Tetra A + 2 poly U at about 60°; the arrow indicates the large increase in absorption at 1625 cm^{-1}.

dipoles or other more specific interactions may also be important. For important recent work on the effect of solvent on the frequency and intensity of carbonyl absorption, the reader is referred to refs. 6–8 and the refs. cited in these articles.

TABLE I

INFRARED SPECTRA IN D_2O SOLUTION

Material	*Temp.*	*νmax (cm⁻¹)*
Poly (A + 2U)	30°	1659; 1699
Poly (A + 2U)	~ 60°	1632; 1657; 1698
Tetra A + 2 poly U	30°	1654; 1697
Tetra A + 2 poly U	~ 60°	1625; 1659; 1697

The spectra were measured with a Beckman IR-7 spectrophotometer at high scale expansion, using matched CaF_2 cells of 0.025-mm path length. The instrument was purged with dry air. The high-polymer solutions were approx. 0.01 *M* in repeating units of AU_2 and 0.1 *M* in NaCl, 0.01 *M* in sodium cacodylate, 0.01 *M* in $MgSO_4$, pH 7.0. The tetra A + poly U solution was approx. 0.005 *M* in repeating units of AU_2, 0.004 *M* in $MgCl_2$, 0.01 *M* in sodium cacodylate, pH 7.2. No NaCl was added.

The high-temperature measurements were made by warming the filled cells to about 60°, measuring the spectra immediately, and then scanning repeatedly as the cells cooled to room temperature.

The above result with the oligonucleotide constitutes additional evidence in support of the three-stranded structure for tetra A + 2 poly U[3].

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An infective deoxyribonucleic acid from bacteriophage φX174

It has recently been demonstrated that bacteriophage øX174 contains an unusual DNA which is composed of a single polynucleotide strand[1]. A nucleic acid of this type is of special interest because enzymic studies have suggested that double-stranded DNA is converted to a single-stranded condition during the course of duplication[2]. The purpose of the present investigation was to devise a method of estimation of the activity of øX174 DNA.

øX174 used in these experiments was generously furnished by Dr. I. TESSMAN of Purdue University. The phage was propagated on *Escherichia coli* strain C in a glycerol–casamino acid medium[3] and purified by differential centrifugation at 6,000 rev./min and 37,000 rev./min. Spheroplasts of *E. coli* and other bacterial strains were prepared by lysozyme treatment[4].

The DNA was extracted from øX174 by suspending the phage in 0.1 *M* NaCl–0.01 *M* phosphate buffer, pH 7.0, at a concentration of 10^{11}–10^{12} phages/ml, and heating at 90° for 10 min. After addition of NaCl to give a concentration of 1 *M*, the solution was shaken with an equal volume of a chloroform–octanol (8:1) mixture to remove protein. The extraction procedures with chloroform were repeated until no gelatinous interface could be observed (at least four times). Ethanol (2 vol.) was added to the aqueous solution and the appearing fibrous precipitate was collected, dissolved in a small volume of 1 *M* NaCl, and re-precipitated with ethanol. DNA thus obtained was dissolved in a sterilized medium.

This preparation of DNA was found to produce many infective phages when incubated with bacterial spheroplasts in a hypertonic sucrose medium. The properties of the preparation differ from those of normal øX174 in the following respects.

(1) The preparation could infect spheroplasts but not normal cells. In addition, spheroplasts of bacteria originally resistant to øX174 (*e.g. E. coli* B, *E. coli* K-12 and

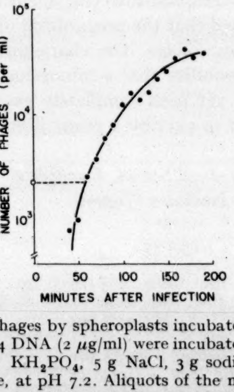

Fig. 1. Production of infective phages by spheroplasts incubated with øX174 DNA. Spheroplasts of *E. coli* B ($2\cdot 10^8$/ml) and øX174 DNA (2 μg/ml) were incubated 37° in a medium containing (per liter) 2.5 g $(NH_4)_2HPO_4$, 1.5 g KH_2PO_4, 5 g NaCl, 3 g sodium glutamate, 3 g glucose, 0.1 g $MgSO_4\cdot 7H_2O$, and 200 g sucrose, at pH 7.2. Aliquots of the mixture were taken at intervals as shown, diluted with saline, and plated on agar layer with a culture of *E. coli* C. Number of infective centers at zero time, indicated by open circle, was determined by plating an aliquot of the mixture on 20% sucrose-agar with *E. coli* C.

Abbreviation: DNA, deoxyribonucleic acid.

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Aerobacter aerogenes 1033) were also infected by the preparation. Thus, in the subsequent experiments, spheroplasts from the resistant strain were used in order to exclude the possibility that the cells remaining after lysozyme treatment were reinfected with any active phages produced by the infected spheroplasts. Fig. 1 shows the formation of infective phages from the DNA preparation in spheroplasts of *E. coli* B.

(2) Infectivity of the preparation was completely destroyed by a brief treatment with DNA-ase, whereas such treatment did not impair the infectivity of intact phage. The results are shown in Table I.

TABLE I

ACTION OF ENZYMES ON THE INFECTIVITY OF ϕX174 DNA

Assay technique as described in Fig. 1.

Treatment of DNA	*Host*	*No. of phages produced*
None	*E. coli* B spheroplast	18,820
DNAase (1 μg/ml)*	*E. coli* B spheroplast	0
Trypsin (100 μg/ml)**	*E. coli* B spheroplast	18,640
None	*E. coli* B normal cell	0
None	*E. coli* C normal cell	0

* Incubated with crystalline DNAase (Sigma Chemical Co.) in 0.02 *M* $MgCl_2$–0.1 *M* tris-(hydroxymethyl)aminomethane buffer, pH 7.2 for 60 min at 37°.

** Incubated with crystalline trypsin (Sigma Chemical Co.) in 0.05 *M* phosphate buffer, pH 7.5 for 60 min at 37°; the enzyme action was stopped by the addition of soybean trypsin inhibitor prior to mixing with spheroplasts.

(3) The preparation had an ultraviolet-absorption spectrum characteristic of nucleic acid and did not contain any detectable amount of protein. It maintained its proper infectivity even after being treated with a high level of trypsin, indicating a distinct difference from the spheroplast-infecting agent from T2 phage[5–8].

Thus evidence was obtained that the preparation of DNA from øX174 possessed the ability to produce infective phage. The characterization of the preparation is now in progress, since the possibility that a minor component in the preparation is required to infection has not yet been completely excluded.

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THE OPTICAL ROTATORY AND VISCOMETRIC PROPERTIES OF γ-POLY-D-GLUTAMATE*

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SUMMARY

The optical rotatory and viscometric properties of the γ-linked polypeptide, γ-poly-D-glutamate, are shown to be markedly dependent on the ionic state of the α-carboxyl side chain group. From the influence of various positively charged ions on the specific rotation of γ-poly-D-glutamate the relative order of their binding has been deduced: $H^+ > Pb^{++} > Cd^{++} > Zn^{++} > Ba^{++} \gg$ guanidinium $\sim Na^+$. The binding of the heavy metals follows quite closely their affinity constants to the carboxylate group in simple aliphatic acids. Optical rotational and viscometric evidence is reported which indicates that the un-ionized polypeptide exists in a hydrogen-bonded hypercoiled form in water.

INTRODUCTION

In recent years considerable effort has been expended in trying to correlate the optical rotatory properties of α-polypeptides, native and denatured proteins with their molecular structure[1-3]. Since the asymmetric carbon atom of the peptide residue forms part of the backbone of the polypeptide chain, changes in optical rotatory behavior will reflect principally modifications in the helical configuration of the polypeptide chain[4,5]. For obvious reasons most interest has been focused on the rotatory properties of α-linked polypeptides. We have examined, however, the optical characteristics of a naturally occurring γ-linked polypeptide in order to compare the behavior of these two types of linkages in polyamino acids.

The γ-linked polypeptide of glutamic acid has been shown to exist in a randomly coiled structure when un-ionized[6,7]. Upon ionization the molecule undergoes considerable expansion as a result of intramolecular electrostatic repulsive forces. It has been demonstrated that α-polypeptides can exist in either a flexible chain or a helix, depending on environmental variables[1,8]. It was of interest, therefore, to determine the behavior of a γ-linked polypeptide in solvents which can lead to the formation of either type of structure.

Abbreviations: GP, γ-poly-D-glutamate; DCA, dichloroacetic acid; EDC, ethylene dichloride.

[*] A preliminary report of the optical rotatory aspects of this paper has been published by the present authors in this journal[41].

In addition we have explored the viscosity changes that occur in some of the solvents in order to ascertain whether any correlation exists between rotation and viscosity in a polypeptide which cannot form an α-helix. In certain proteins, *e.g.* serum albumin[9], ovalbumin[10–12], γ-globulin[13,14], and α-polypeptides[15], changes in rotation are paralleled by those in viscosity while in other proteins such as RNAse in H_2O–chloroethanol solutions[16] or gelatin in various neutral salt solutions[17] little or no relationship was found.

METHODS AND MATERIALS

We are greatly indebted to Dr. C. B. THORNE for all samples of γ-poly-D-glutamate. This material was obtained from the capsule of *B. anthracis* and consists of only D-glutamic acid[18] linked through the γ-carboxyl and α-amino group[19,20]. Unless otherwise stated all the data was obtained with a preparation No. 74. This material has been characterized by physical methods and has a molecular weight of 250,000 as determined by sedimentation and diffusion. In acid the sedimentation pattern shows a single peak with considerable spreading. The material is therefore heterodisperse with a mean sedimentation value in acid, at zero protein concentration, of 5.8 S.

Lithium bromide was decolorized with norite and then recrystallized several times. Guanidine hydrochloride was also recrystallized. The remaining salts were reagent grade and were used directly.

Viscosities were measured in an Ostwald viscometer held in a fixed position in a water bath regulated to ± 0.01°. Kinetic energy corrections were considered insignificant and were not applied. The specific viscosity, ηsp., was defined as $(t - t_0)/t_0$, where t and t_0 are the flow times in the solution and solvent respectively. The concentration, c, is in g/100 ml. All solutions were centrifuged at 20,000 × g for 30 min.

Optical rotation and rotatory dispersion measurements were performed with a Rudolph Model 80 photoelectric polarimeter. Measurements of the temperature coefficients were made initially with a Keston polarimeter attachment to the Model DU Beckman spectrophotometer. Several of the curves were verified subsequently in a thermostatted Rudolph polarimeter.

All the optical rotational data conformed to a single term Drude equation. The critical wavelength (λ_c) was determined from the slope of a plot of $[\alpha]\lambda^2 = [\alpha]\lambda^2_c + k$. Owing to the high molecular weight of preparation No. 74, solutions were fairly turbid and measurements could only be made with satisfactory precision between 700 and 400 mμ. A few experiments were made with another preparation (No. 75), of lower molecular weight ($s^0_{20,w} = 2.8$) where dispersion data could be obtained to wavelengths of ~ 350 mμ. The rotational constants of this preparation agreed with those of No. 74 in all respects. The observed rotational values have been corrected for the refraction of the solvent to that of water by the Lorentz factor $\frac{(n^2_w + 2)}{(n^2_s + 2)}$ and specific rotations are stated on the basis of the weight of the Na salt of GP. The temperature was between 23 and 25°.

At pH values below ~ 2.0 GP is completely un-ionized and this form will be described as HGP. At pH values above ~ 7 GP is 100% neutralized and will be referred to as NaGP. When evaluating properties of GP at intermediate levels of ionization, the degree of ionization will be explicitly stated. The degree of ionization was determined by a potentiometric titration curve of GP under conditions identical with those cited for the particular property under investigation. Potentiometric titration curves of GP, at several ionic strengths, have been reported elsewhere[6].

The optical rotatory properties of GP

Effect of pH in salt-free solutions: The specific rotational values of GP from pH 2.9 to 7.2 in salt-free solution are illustrated in Fig. 1. When the specific rotation was plotted in accordance with the Drude equation ($[\alpha]\ \lambda^2$ *vs.* $[\alpha]$) linear relationships were always found. The curves in Fig. 1 are theoretical and are based on values of λ_c and k determined from Drude plots of the experimental data which are shown by the points. Values of λ_c less than ~ 200 mμ, especially when coupled with small values of $[\alpha]$, were particularly difficult to determine precisely. Two illustrative examples of the dependence of $[\alpha]$ on smaller values of λ_c, *i.e.*, $i100$ mμ and 0 are shown in Fig. 1. In the solid line at pH 3.60, λ_c was equal to $i140$ mμ. The k values have been adjusted so that all three curves intersect at 500 mμ*.

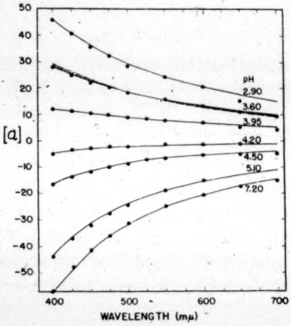

Fig. 1. The rotatory dispersion of GP as a function of pH. The curves are theoretical and based on the simple form of the Drude equation. The points are experimental. The dashed curve at pH 3.6 employed a value of $\lambda_c = i100$ instead of $i140$ as in the full curve. The dash-dot curve is for $\lambda_c = 0$. GP $\cong$ 1 %. Temperature, 23 to 25°.

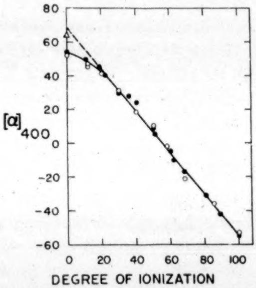

Fig. 2. Variation of specific rotation (at 400 mμ) with the degree of ionization of GP; ●, preparation No. 74; ⊙, No. 75. △, represents the optical rotation of HGP in 6.0 M guanidine from pH 2.2 to 1.5.

When the values of $[\alpha]$ at 400 mμ were plotted against the degree of carboxyl ionization a linear dependence was observed from 100 to 20 % ionization. At smaller values of ionization $[\alpha]$ increased somewhat less rapidly, as seen in Fig. 2.

Though $[\alpha]$ varied smoothly with ionization, values of the Drude constant, λ_c, showed an abrupt transition between ~ 50 and 60 % ionization. The values of λ_c became imaginary when $[\alpha]$ changed sign. The variations in λ_c with degree of ionization are shown in Fig. 3. Above 60 % ionization λ_c values are in the range of those reported for native and denatured proteins. Below 30 % ionization the Drude constant was indistinguishable from zero. Between 30 and 60 % it was imaginary. The abrupt change observed in λ_c between 50 and 60 % ionization has not been seen in any other property of GP evaluated to date.

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* The significance of the imaginary values of the Drude Dispersion constant, λ_c, is not apparent. It is perhaps more realistic to consider the Drude equation as an empirical formula where imaginary values of λ_c are observed. It is possible that some small systematic error (perhaps due to an optically active absorbing impurity) has distorted the data to produce the imaginary value of λ_c. On the other hand a slightly modified Drude equation could readily eliminate the imaginary value of λ_c.

Effect of ionic strength (NaCl): The rotatory constants for NaGP in NaCl solutions between 0 and 5.0 *M* are compiled in Table I. With the exception of the data for 5 *M* NaCl the values are similar to those reported for denatured proteins. The increase in rotation that occurs at high concentrations of NaCl is in the same direction as observed by reducing the degree of ionization of NaGP with acid. Therefore the influence of NaCl on the rotation of GP can be explained by the binding of Na^+ ions. The binding of Na^+ by synthetic polyelectrolytes is a well known phenomenon[21-23]. In contrast to the influence of NaCl on NaGP, the rotatory dispersion curve of HGP was unaffected by 1.0 *M* NaCl.

Urea, guanidine and LiBr: Both urea and guanidine are well known denaturing reagents[5,10-12]. As seen in Table I, 8.0 *M* urea produced very little alteration whereas

TABLE I

OPTICAL ROTATORY PARAMETERS OF NaGP IN VARIOUS SOLVENTS

Solvent	*Molarity*	$[\alpha]_{100}$	λ_c *(mμ)*
NaCl	0	—56°	220
	0.01	—55	211
	0.10	—48	208
	1.00	—21	204
	5.00	+14	~150
Urea	8.0	—35	208
Guanidine	3.0	+21	~0
	6.0	+36	170
LiBr	8.0	—15	280
		$[\alpha]_{125}$	
DCA		—30	270
DCA-EDC (53:47)		—19	270

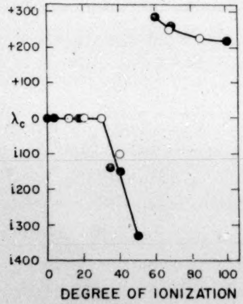

Fig. 3. Variation of the Drude dispersion constant (λ_c) with the degree of ionization of GP; ●, preparation No. 74; ⊙, No. 75.

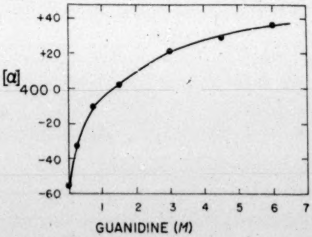

Fig. 4. Effect of guanidine on the specific rotation (at 400 mμ) of NaGP. NaGP concentration was 1 %.

3.0 and 6.0 M guanidine had marked effects on the rotatory constants of NaGP. Indeed as shown in Fig. 4 an increase of 92° was observed in $[\alpha]_{400}$ in 6.0 M guanidine. The specific rotations were found to be independent of NaGP concentration (from 1.00 to 0.25 % NaGP) in 0.25 M and 4.5 M guanidine*.

When GP was uncharged, 6.0 M guanidine increased its specific rotation by 10°. A value of 68 ± 2° was obtained from pH 2.2 to 1.5 in 6.0 M guanidine solutions. This value of $[\alpha]$ is close to that which would be observed if the linear portion of the curve shown in Fig. 2 were extrapolated to zero degree of ionization. In this pH range HGP tends to become turbid in water, indicating a limited solubility. In 6.0 M guanidine HGP solutions were quite clear. The specific rotation of HGP was independent of concentration, from 0.25 to 1.0 %, in water or in 5.0 M guanidine at pH 1.75.

It has been shown by HARRINGTON AND SCHELLMAN[25] that moderate concentrations of LiBr, by reducing the activity of water, tend to enhance the enthalpy of a hydrogen bond and thereby promote hydrogen bonding in proteins. Although a γ-linked polypeptide, such as GP, cannot form a Pauling-Corey α-helix, it can engage in extensive intramolecular hydrogen bonding either between the backbone CO and NH groups, side chain carboxyl groups, or backbone and side chain groups.

HGP was soluble (1 % solutions were slightly opalescent) in 4.8 M LiBr and quite insoluble in 8.0 M LiBr. However, NaGP was readily soluble in 8.0 M LiBr and insoluble in 9.6 M LiBr. In 8 M LiBr the specific rotation of NaGP was $-15°$ and λ_c was 280 mμ. The directional change of both these values, when compared to their values in water (pH ~7), resemble those found in α-polypeptides, native and denatured proteins in non-interacting solvents[1,25]. When NaGP in 8 M LiBr was acidified to a point just prior to precipitation (about 25 % of the acid needed to neutralize all the charged groups was added) $[\alpha]$ increased to $+8°$ and λ_c was *i*150 mμ. The rotational properties of HGP in 4.8 M LiBr were similar to those observed in water.

Non-aqueous solvents: YANG AND DOTY[1] have shown that certain organic solvents are good hydrogen-bond-breaking reagents while others, by providing a non-interacting milieu, tend to strengthen hydrogen bonds. Thus, by working with mixtures of DCA and EDC the α-helical content of a protein or polypeptide, as reflected by changes in both $[\alpha[$ and λ_c, may be diminished or augmented by varying the proportions of the two solvents. NaGP was quite soluble in DCA. Since DCA is a much stronger acid than glutamic, GP should be un-ionized in this solvent. The rotatory constants reported for DCA in Table I, however, are quite different from those found for HGP in water or even in 5 M guanidine solutions. When EDC was added to a DCA solution to a point just short of precipitating the GP (DCA–EDC 53:47), not much change was observed in either $[\alpha]$ or λ_c (see Table I).

Since alcohol (ethyl) has a considerable influence on the viscosity of HGP[26], its effect on the rotatory properties was also investigated. In both 25 and 50 % mixtures of methyl alcohol and water (v/v) the dispersion curves were unchanged from that observed in water.

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* If we assume that the change in specific rotation of NaGP is proportional to the amount of guanidine bound, then at about 1 M guanidine half the sites are occupied. As a crude approximation this results in $K_A \cong 1$. TANFORD[24] found that the affinity constant (K_A) of acetate for guanidine is < 0.5. Electrostatic effects could readily account for the difference in constants.

The viscometric properties of GP

Effect of ionization: The dependence of the viscosity of GP on its charge, at several ionic strengths is shown in Fig. 5. The viscosity increased monotonically with the degree of ionization. At all three ionic strengths an initial lag occurred in the early stages of ionization. Subsequently the viscosity increased almost linearly until ionization was complete.

The concentration and ionic strength dependence of the viscosity of NaGP: The effect of NaGP and NaCl concentration on the specific viscosities of NaGP is illustrated in Fig. 6. Similar data for HGP appears in Fig. 7. It is evident that even 5 *M* NaCl fails to reduce the viscosity and hence the dimensions of NaGP to that of HGP. With other polyelectrolytes 1 *M* salt usually suffices to bring the viscosity of the ionized form close to that of the un-ionized molecule[27, 28].

In Fig. 8 both the intrinsic viscosity and specific rotation of NaGP are plotted as a function of NaCl concentration. It is evident that the intrinsic viscosity and optical rotation depend on NaCl concentration in quite different ways. In low salt concentrations the viscosity falls rapidly as ionic strength increases whereas the optical rotation shows but little variation. In high salt concentrations the rotation changes considerably while the viscosity is much less affected.

Urea, guanidine and LiBr: It might be postulated that the very large values found

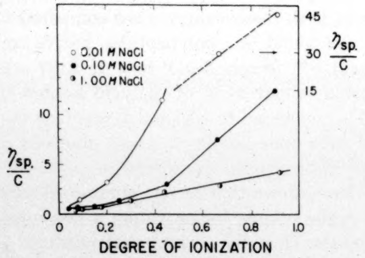

Fig. 5. Influence of the degree of ionization on the specific viscosity of GP at several ionic strengths. GP ≅ 0.125%. Temperature, 28.0°.

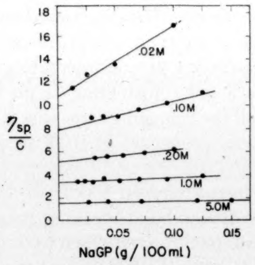

Fig. 6. Concentration dependence of specific viscosity of NaGP at several ionic strengths. Temperature, 28.0°.

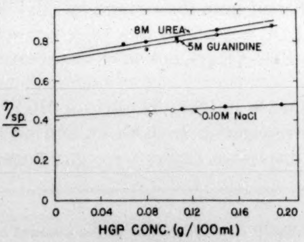

Fig. 7. Concentration dependence of the specific viscosity of HGP in water (at pH 1.9 and 1.55) and in 8 *M* urea and 5 *M* guanidine, at pH 1.55.

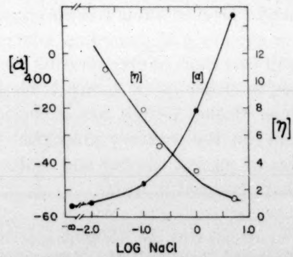

Fig. 8. Effect of NaCl on the specific rotation and intrinsic viscosity of NaGP.

for the intrinsic viscosity of NaGP (in 0.1 *M* NaCl) might be due to intramolecular hydrogen bonding between charged carboxylate and neighboring NH groups. Ring formation of this type would result in a reduction in rotational freedom with considerable stiffening of the polypeptide chain. In order to check on this possibility the viscosity of NaGP (in 0.10 *M* NaCl) was determined in 8 *M* urea. In this solvent the intrinsic viscosity was close to 10 and thus somewhat greater than the value of 7.9 found in the absence of urea. Therefore intramolecular hydrogen bonding would not appear to be of significance to the structure of NaGP. Guanidine is usually more effective, at comparable concentrations, than urea in rupturing hydrogen bonds. Since guanidine at neutral pH values is a cation it would contribute to the ionic strength of the solution and therefore would not be likely to provide as unambiguous a result as urea.

TABLE II

INTRINSIC VISCOSITY OF GP IN VARIOUS SOLVENTS

Form of GP	*Molarity Solvent*	*pH*	*[η]*
HGP	0.10 NaCl	1.90	0.41
	0.10 NaCl	1.55	0.41
	5.0 Guanidine	1.55	0.70
	8.0 Urea–0.10 NaCl	1.55	0.72
NaGP	0.10 NaCl	7.0	7.9
	8.0 Urea–0.10 NaCl	7.0	10.0
	8.0 LiBr		0.66

It is possible that the disparity in viscosity between NaGP in strong salt and HGP results from hypercoiling in HGP due to random intramolecular hydrogen bonding. This possibility was tested therefore by measuring the viscosity of HGP in urea and guanidine. As seen in Fig. 7 there was about a 50% increase in the viscosity of HGP in either 8.0 *M* urea or 5.0 *M* guanidine. Since both reagents produced comparable effects it would appear that the increase in viscosity resulted from the rupture of intramolecular hydrogen bonds which cross-link the linear polypeptide.

The viscosity of NaGP in 8 *M* LiBr was only somewhat greater than that observed for HGP in dilute acid. Since GP is still partially ionized in this solvent the low value of its intrinsic viscosity is particularly noteworthy.

The effect of temperature on optical rotation

In a random coil polymer an increase in temperature will enhance the rotational freedom of the molecule which should result in a decrease in the numerical value of the specific rotation[29]. The influence of temperature on the rotatory behavior of GP in several salt solutions is shown in Fig. 9. The decrease in rotation that occurs with HGP (in water or 0.10 *M* NaCl) at elevated temperatures is consistent with a structure which is not constrained to a specific configuration. An increase in rotation would be expected if intramolecular hydrogen bonds were broken at higher temperatures. However, this effect could be eclipsed by the negative temperature coefficient of rotation found for coiled polymers.

The increase in levorotation observed with NaGP in either 0.01 *M* or 5.0 *M* NaCl was unforeseen. A similar curve displaced by about 20° above the 0.01 *M* NaCl, was obtained with 90% ionized GP in 0.10 *M* NaCl. The increase in 5.0 *M* NaCl, however, may be a reflection of an increase in charge resulting from a reduction of Na^+ binding by GP at the higher temperatures.

In comparison with the other curves shown in Fig. 9 that of NaGP in 8 M LiBr was quite distinctive in that it showed only very minor changes in rotation with temperature. In this respect NaGP behaved in much the same way as α-linked polypeptides and proteins in this salt. According to HARRINGTON AND SCHELLMAN[25] this lack of a temperature coefficient is due to the greatly enhanced strength of hydrogen bonded groups which tends to lock the molecule into a specific configuration which is resistant to thermal agitation at the temperatures normally studied.

The interaction of heavy metals with NaGP

Though very high concentrations of NaCl were required to reduce significantly the viscosity of NaGP, certain heavy metallic ions were very effective at rather low concentrations. The influence of several of these cations on the viscosity of NaGP is illustrated in Fig. 10. In order to keep the ionic strength approximately constant, all solutions contained either 0.10 M NaCl or KNO_3. The data for all four curves extend close to the region where GP becomes insoluble. It is evident that all of these salts are vastly more efficient than NaCl in reducing the viscosity and hence the effective volume of GP. In fact the relative effectiveness of the four cations shown in Fig. 10 parallels their relative binding affinities for the carboxylate anion in simple carboxylic acids[30].

Since the optical rotation of GP has been found to vary regularly with the binding of protons it was not surprising to find that it also increased uniformly with

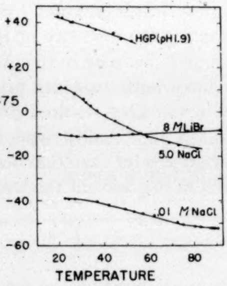

Fig. 9

Fig. 9. Temperature dependence of the specific rotation of GP at 475 mμ. GP concentration ≅ 1.0%.

Fig. 10. Effect of heavy metal salts on the specific viscosity of NaGP. All solutions contained 0.10 M KNO_3. Reagents were $Pb(NO_3)_2$, $Cd(NO_3)_2$, $ZnSO_4$, $BaCl_2$. NaGP ≅ 0.098%, Temperature, 28.1°.

Fig. 11. Effect of heavy metal salts on the specific rotation of NaGP. Reagents were the same as used in Fig. 10. The lead and zinc solutions contained 0.10 M KNO_3. The cadmium and barium solutions contained 0.10 M NaCl. NaGP ≅ 1.0%. Temperature, 23–25°.

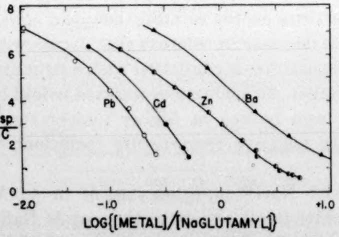

Fig. 10

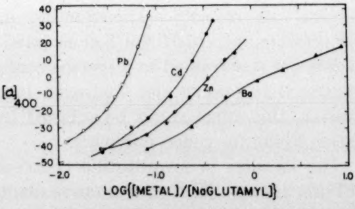

Fig. 11

the binding of heavy cations. The influence of Cd^{++}, Zn^{++} and Ba^{++} on the specific rotation paralleled their effect on the viscosity of NaGP. However, Pb^{++} appears to be more efficacious in modifying the rotatory than the viscosity properties. As a measure of the relative effectiveness of the metallic ions we have listed in Table III the amount of metal needed to increase the specific rotation from —48° to 0°. The affinity constants for the carboxylate anion are also shown. It is apparent that the relative influence of the metal corresponds quite well with its affinity for the simple carboxylate anion. This correspondence would tend to preclude any large effect of the peptide backbone on the interaction of GP with the investigated cations.

The binding of these ions to NaGP has been demonstrated also by potentiometric and electrophoretic methods[26].

TABLE III

CORRELATION BETWEEN THE AFFINITY OF CERTAIN METAL SALTS TO ACETATE WITH THAT TO NaGP

Salt	*log (M^{++}/GP)* *	*Δ*	*log K*	*Δ^1*
$Pb(NO_3)_2$	—1.32		2.0	
		0.59		0.7
$Cd(NO_3)_2$	—0.73		1.3	
		0.30		0.27
$ZnSO_4$	—0.43		1.03	
		0.58		0.64
$BaCl_2$	+0.15		0.39	

[*] This expression indicates the amount of heavy metal ion (M^{++}) required to increase the specific rotation of NaGP from —48 to 0 at 400 mμ. GP refers to the glutamyl residue of molecular weight 129. The concentration of NaGP was close to 1%. Δ shows the numerical difference in the logarithmic function between neighboring salts. Δ^1 is the difference in the logarithm of the affinity constants between neighboring salts. K is the affinity constant of the metal ion for the carboxylate group and is taken from Table VII of GURD AND WILCOX[30].

DISCUSSION

It is clear from the continuous change in specific rotation with degree of carboxyl ionization that GP shows rotatory properties at variance with that of α-polypeptides and proteins. In the latter cases optical rotatory values are usually fairly independent of moderate changes in ionization[31] unless structural changes occur simultaneously[9-14]. It is well known that the specific rotation of amino acids and small polypeptides is markedly influenced by the charged state of the ionogenic groups[32]. However, in high polymeric α-polypeptides, composed of single amino acids, the influence of terminal groups rapidly vanishes as the number of residues becomes large and consequently the mean residue rotation becomes constant[3, 33]. In GP the situation is peculiar since the ionizable side chain carboxyl groups are located α to the asymmetric carbon atom and consequently are the predominant influence in determining the rotatory behavior of GP in aqueous solvents. In proteins the charged groups, other than terminal, and with the exception of aspartic acid which is β, are never closer than the γ position to the asymmetric center. Hence their influence on the asymmetric carbon atom will be diminished materially.

The influence of metal ions, which are known to bind strongly to carboxylate anions, has demonstrated that the relative effectiveness of these ions in increasing the specific rotation of NaGP is indeed related to their binding constants. The binding of cations can therefore influence the rotational properties of GP in much the same way as the binding of protons. The rather large amounts of Na^+ required to increase the rotation (to some arbitrary level), when compared with the relatively small amounts of strongly bound ions, attests to the weakness of its binding to GP. That guanidine is also bound to the carboxylate group is evident from the fact that urea, which shows comparable denaturing ability, has only a small effect on the dispersion characteristics of NaGP. Though the changes in $[\alpha]$ observed in NaCl and guanidine solutions are comparable to those produced by changes in ionization, the values of λ_c vary for identical values of $[\alpha]$. This is not surprising when one considers the chemical differences between the bound ions. Appreciable differences in the effects of salts on the optical rotatory properties of amino acids have been noted[34].

The binding of protons and cations to NaGP therefore is measurable by changes in optical rotation. It is apparent that the charge on the anionic GP molecule will be reduced as a consequence. Since the dimensional properties of polyelectrolytes are a function of net charge as well as ionic strength[35–37] the binding of oppositely charged ions should be reflected by modifications in the viscometric and other colloidal solution properties[21–23].

In dilute salt solutions strongly bound ions will attenuate the viscosity of NaGP by their influence on the net polymeric charge whereas weakly bound ions will depress the viscosity principally by contributing to the ion atmosphere. Consequently, viscosity and rotatory changes will approximately parallel each other when they result from a change in charge as with the heavy metal salts investigated. On the other hand when viscosity changes ensue from a build-up in the ion cloud, as with weakly bound ions such as NaCl and guanidine salts, major viscometric changes can occur without concomitant effects on optical rotatory behavior.

The fact that the linear relationship between specific rotation and degree of ionization extends over most (20–100% ionization) but not all of the curve, suggests that some structural factor may be influential in modifying the rotational properties in the first 20% of ionization of GP. A similar disparity is evident in the viscosity curves shown in Fig. 5. The rise in viscosity that occurs between 0 and 20% ionization is decidedly less than the change observed in a similar portion of the remainder of the curve. In fact, above $\sim$30% ionization there is a linear increase in specific viscosity with ionization whereas, below, it is convex towards the abscissa. KATCHALSKY[38] has reported similar viscometric behavior with synthetic α-polyaspartic acid. He attributed the initial lag in viscosity to the inability of the electrostatic field produced by the low degree of ionization to overcome intramolecular cohesive forces due to hydrogen bonding between segments of the polymeric chain. If this type of cross-linking is appreciable, the configuration of GP at low degrees of ionization would assume that of a hypercoiled form. As the charge increases, the electrostatic repulsive forces outweigh the cohesive forces and the molecule expands rapidly. The expansion now depends on the balance between electrical stretching forces and Brownian contractile forces.

The increase in viscosity of HGP found in urea and guanidine solutions suggests an unfolding of the HGP molecule which depends on the rupture of hydrogen bonds. The failure of the specific rotation at low values of ionization, to fit the linear relation observed above 20%, suggests a gradual modification in the structure of GP. As the net charge of GP is reduced by acid, the nature of the solute–solvent interaction is altered in accord with the concept of water acting as a good solvent at high degrees of ionization and as a poor solvent below $\sim 20\%$. Direct thermodynamic support is provided by light scattering data of the fully ionized and un-ionized forms of GP. In the absence of salt the second virial coefficient is very large for NaGP and very small for HGP[6]. When HGP was dissolved in a good solvent, such as a 5.0 M guanidine solution, the specific rotation increased to the value HGP presumably would have if water had remained an effective solvent for HGP. Similar conclusions have been reached by SILBERBERG *et al.*[39], who have presented evidence that (un-ionized) polymethacrylic acid is hypercoiled in dilute HCl solutions as a result of intramolecular hydrogen bonding between carboxyl groups. MOREWETZ[40] has presented data on styrene–methacrylic acid copolymers in organic solvents which shows that the intrinsic viscosity is inversely related to the degree of hydrogen bonding.

The optical rotatory and solubility behavior indicate that NaGP in 8 M LiBr is partially ionized. From the rotational data it cannot be ascertained with any large degree of confidence whether the observed changes are due to charge neutralization effects or to other characteristics of LiBr. When GP is 65 % ionized both $[\alpha]$ and λ_c in water are about the same as the values observed for NaGP in 8 M LiBr. The properties of the acidified 8 M LiBr solution corresponds approximately to GP at 50 % ionization.

The similarity in rotatory constants does not however carry over to the viscometric behavior. The intrinsic viscosity of NaGP in 8 M LiBr is substantially less than that of 65 % ionized GP even when in 1 M NaCl. It would appear therefore that intramolecular hydrogen bond formation is enhanced in 8 M LiBr. The indifference of $[\alpha]$ to temperature in this solvent lends further support to this point of view. Consequently it seems reasonable to assume that both HGP in water and NaGP in 8 M LiBr have hypercoiled forms. They do not however approach the highly condensed forms of many globular proteins.

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DIE BESTIMMUNG DES MOLEKULARGEWICHTS AUS ZWEI PUNKTEN UND DEM MAXIMUM DER GRADIENTENKURVE DER ULTRAZENTRIFUGE

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(Eingegangen am 18 März, 1960)

SUMMARY

The determination of the molecular weight from two points and the maximum of the ultracentrifugal gradient curve

The theory of Brownian motion in the centrifugal field, derived previously, provides a new method, the "Zweipunkt-Methode", (2PM), for the determination of the sedimentation coefficient *s* as well as the diffusion coefficient D from the maximal gradient and two points of a single experimental gradient curve obtained by means of the usual optical systems of the ultracentrifuge.

The experimental conditions and the verification of this method are discussed for three various substances with molecular weights between 20,000 and 150,000. The obtained results in case of these substances are in good agreement with the data given in the literature.

The mean error of the values of s and D for medium molecular weights (~ 100,000), obtained at optimal conditions of experiment and evaluation, was 1 to 3 %, *i.e.* only one third of the error in the case of the three-points-method mentioned in a previous paper. For small molecular weights the mean error does not exceed 8 %. The variations of s/D are less than 5 % for medium molecular weights, for small ones they approach 20 % as a maximum.

Comparing the various former methods with the 2PM it seems that the 2PM has some advantages in the case of ideal systems of homogeneous particles; on the other hand the 2PM seems to be suitable to detect qualitatively the heterogeneity of strong polydisperse systems.

EINLEITUNG

Die mathematische Analyse der Gradientenkurve in der Ultrazentrifuge[1,2] liefert Beziehungen für die Konzentration und den Konzentrations- bzw. Brechungsindex-Gradienten einer in der Ultrazentrifuge rotierenden Lösung von Makromolekülen, die neue Möglichkeiten zur Bestimmung des Sedimentations- und Diffusionskoeffi-

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Abkürzungen: SM, Svedberg-Methode; MGM, Maximalgradienten-Methode; 3PM, Dreipunkt-Methode; 2PM, Zweipunkt-Methode. Die früher formulierte graphische "Two-points-method" ist von der vorliegenden 2PM zu unterscheiden.

zienten, bzw. ihres Quotienten s/D, d.h. zur direkten Molekulargewichtsbestimmung eröffnen[2-5].

Die bisher vorgeschlagenen Wege erweisen sich insofern als nicht ganz befriedigend, als auf Grund der unzureichenden optischen Hilfsmittel, die die Empfindlichkeit der mathematischen Analyse nicht annähernd auszunutzen gestatten, Fehler in s/D zwischen $> 6\%$ (3PM) und 17 % (MGM) auftraten.

Im folgenden wird nun eine aus den gleichen Voraussetzungen gewonnene Methode mitgeteilt, die bei Berücksichtigung bestimmter experimenteller Vorbedingungen mit den gebräuchlichen Hilfsmitteln der PHILPOT-SVENSSON-Optik weniger stark mit systematischen und subjektiven Fehlern behaftet ist, sodass mit guter Reproduzierbarkeit Werte von s, D und daraus M berechnet werden können, die für die untersuchten Standard-Substanzen mit den Angaben in der Literatur gut übereinstimmen.

Theoretische Grundlage

Wenn eine ideale, monodisperse und nicht elektrolytische, bzw. beim isoelektrischen Punkt (IP) befindliche Lösung in einer Zelle mit "künstlicher Grenze" (synthetic boundary) bei konstanter Geschwindigkeit ω und konstanter Temperatur rotiert und wenn die Schichtzonen und die Gradientenkurve weder durch den Meniskus noch durch den Boden der Zelle beeinflusst sind, lässt sich der Gradient des Brechungsindex der Lösung $\partial n/\partial r$ bei jedem Abstand r vom Rotationszentrum und zu jeder Zeit t vom Beginn des Versuchs mit Hilfe der Näherungsgleichung

$$\frac{\partial n}{\partial r} = \frac{(\Delta n_0)\,\mathrm{e}^{-2\beta t}}{(4\pi D\alpha t)^{\frac{1}{2}}} \cdot \mathrm{e}^{-\frac{(r_0\,\mathrm{e}^{\beta t} - r)^2}{4D\alpha t}} \cdot \left(\frac{r_0\,\mathrm{e}^{\beta t}}{r}\right)^{\frac{1}{2}} \tag{1}$$

ausdrücken[2], mit: $\beta = s\omega^2$; s, Sedimentationskoeffizient; $\alpha = (\mathrm{e}^{2\beta t} - 1)/(2\beta t)$; r_0, Abstand der Schichtgrenze vom Rotationszentrum zur Zeit $t = 0$; D, Diffusionskoeffizient und Δn_0, Differenz der Brechungsindices von Lösung und Lösungsmittel zur Zeit $t = 0$.

Da der Faktor $\left(\frac{r_0\,\mathrm{e}^{\beta t}}{r}\right)^{\frac{1}{2}}$ den im übrigen symmetrischen Ausdruck der Gl. (1) verzerrt, ersetzen wir den Gradienten $\frac{\partial n}{\partial r}$ durch eine neue Funktion $w = r^{\frac{1}{2}}\left(\frac{\partial n}{\partial r}\right)$, die eine GAUSSsche Kurve darstellt. ln w lässt sich dann folgendermassen formulieren:

$$\ln w = k - \frac{(r_0\,\mathrm{e}^{\beta t} - r)^2}{4D\alpha t}, \tag{2}$$

wobei $k = (\Delta n_0)\,\mathrm{e}^{-2\beta t}\left(\frac{r_0\,\mathrm{e}^{\beta t}}{4\pi D\alpha t}\right)^{\frac{1}{2}}$ von r unabhängig ist. Aus zwei beliebigen Punkten der Gradientenkurve $\mathrm{P}_i\left[r_i; \left(\frac{\partial n}{\partial r}\right)_i\right]$ und $\mathrm{P}_j\left[r_j; \left(\frac{\partial n}{\partial r}\right)_j\right]$ lassen sich nun nach Gl. (2) Ausdrücke für die entsprechenden ln w_i und ln w_j ableiten, aus denen wir die folgende Funktion gewinnen:

$$g_{ji} = \frac{\ln w_j - \ln w_i}{r_j - r_i} = \frac{(r_0\,\mathrm{e}^{\beta t} - r_i)^2 - (r_0\,\mathrm{e}^{\beta t} - r_j)^2}{4D\alpha t\,(r_j - r_i)} = \frac{2\,r_0\,\mathrm{e}^{\beta t} - r_j - r_i}{4D\alpha t}. \tag{3}$$

Nun gilt für den Maximalgradienten die Approximation[2]

$$H \approx \frac{(\Delta n_0)\,\mathrm{e}^{-2\beta t}}{(4\pi D\alpha t)^{\frac{1}{2}}}; \tag{4}$$

daraus folgt

$$4D\alpha t = \frac{(\Delta n_0)^2 \, e^{-4\beta t}}{\pi H^2}, \tag{5}$$

sodass Gl. (3) in

$$f_{ji} = \frac{g_{ji}\,(\Delta n_0)^2}{2\pi H^2} = r_0 \cdot e^{5\beta t} - \bar{r}_{ji} \cdot e^{4\beta t} \tag{6}$$

transformiert werden kann, wobei $\bar{r}_{ji} = \frac{r_j + r_i}{2}$ den mittleren Radius der Punkte *j* und *i* darstellt.

Da bei nicht zu langer Versuchsdauer die Ungleichung

$$1 >> 5\beta t >> (5\beta t)^2 >> \ldots$$

gilt, kann in erster Näherung

$$e^{5\beta t} \approx 1 + 5\beta t \text{ und } e^{4\beta t} \approx 1 + 4\beta t$$

substituiert werden. Gl. (6) geht dann über in

$$f_{ji} = (r_0 - \bar{r}_{ji}) + (5r_0 - 4\bar{r}_{ji})\,\beta t \tag{7}$$

und endlich in

$$\beta t = \frac{f_{ji} + (\bar{r}_{ji} - r_0)}{5r_0 - 4\bar{r}_{ji}} \tag{8}$$

Diese Beziehung liefert wegen $\beta = s\omega^2$ unmittelbar den Sedimentationskoeffizienten *s*.

Der Diffusionskoeffizient *D* resultiert in analoger Weise aus Gl. (3), indem bei kurzer Versuchsdauer

$$\frac{1}{\alpha} \approx e^{-\beta t} \approx 1 - \beta t$$

gilt, sodass Gl. (3) sich vereinfacht zu

$$g_{ji} = \frac{r_0 - \bar{r}_{ji}\,(1 - \beta t)}{2Dt} \tag{9}$$

Daraus

$$D = \frac{r_0 - \bar{r}_{ji}\,(1 - \beta t)}{2\, g_{ji}\, t} \tag{10}$$

EXPERIMENTELLER TEIL

Die im vorigen abgeleiteten Gleichungen zur Bestimmung des Sedimentations- und Diffusionskoeffizienten aus der Gradientenkurve in der Ultrazentrifuge wurden im Molekulargewichtsbereich zwischen 20,000 und 150,000 in der Spinco-Ultrazentrifuge Modell E experimentell geprüft.

Die dabei als notwendig erkannten experimentellen Voraussetzungen seien zusammen mit den angewandten Messmethoden, soweit sie von der Norm abweichen, im folgenden kurz angegeben.

Experimentelle Voraussetzungen

Die synchrone Bestimmung von *s* und *D* in der Ultrazentrifuge stellt auf Grund der grossen Empfindlichkeit der BROWNschen Bewegung gegen äussere Störungen an die Mechanik, Elektronik und Optik der Ultrazentrifuge hohe Anforderungen. Diese beziehen sich (a) auf die Erschütterungsfreiheit des Systems, (b) auf die Konstanz der Winkelgeschwindigkeit, (c) auf den Ausschluss von Präzessionsbewegungen des Rotors, (d) auf maximale Temperaturkonstanz, (e) auf die Exaktheit der Angabe des PHILPOT-Winkels φ und (f) auf optimale Verhältnisse bei der Abbildung der Gradientenkurve.

Alle diese Voraussetzungen, mit Ausnahme der letzten, können in der hier zugrundeliegenden Ultrazentrifuge unter bestimmten Bedingungen als hinreichend erfüllt angesehen werden. Die Einschränkung bzgl. der Abbildung der Gradientenkurve beruht dabei auf der systembedingten mangelhaften Lokalisierbarkeit der "theoretischen" Gradientenkurve in dem vom optischen System entworfenen photographischen Bild.

Die exakte Theorie der BROWNschen Bewegung im Zentrifugalfeld bedürfte zu ihrer Bestätigung ebenso wie zu ihrer allgemeinen Anwendung einer sehr schmalen scharfen Gradientenkurve und Basislinie, die derzeit nur mit einer gewissen Willkür dem photographischen Bild entnommen werden kann, etwa durch Konstruktion der "Mittelpunkts-Kurve" zwischen äusserer und innerer Kante der Abbildung des Diaphragmas (Schneide, Phasenplatte, Spalt, Draht, Phasenkante) oder durch Wahl der die Diaphragma-Abbildung nach unten begrenzenden Kurve ("Tangenten-Kurve"). Die im folgenden mitgeteilten Messungen werden zeigen, dass die hier diskutierte Methode gegen derartige Fehlereinflüsse verhältnismässig unempfindlich ist, sodass sie mit allen heute gebräuchlichen optischen Hilfsmitteln angewandt werden kann.

Messmethode

Die für die hier mitgeteilten Messungen erforderliche Stabilität von Temperatur und Winkelgeschwindigkeit, sowie die Herabminderung der Präzession des Rotors lässt sich unter bestimmten experimentellen Bedingungen erreichen.

1. Es empfiehlt sich dabei, auf die Verwendung des Kühlaggregates oder der Thermoregulierung während der Messung zu verzichten, da diese Einrichtungen die voraussetzungsgemäss kurzen Messungen zu stören vermögen. Eher erscheint es ratsam, die Ergebnisse auf die gewünschte Temperatur zu korrigieren oder geraume Zeit vor Beginn des Versuches das System auf diese Temperatur zu bringen.

2. Bei der Wahl der Winkelgeschwindigkeit hat man unter Berücksichtigung der Grössenordnung des Molekulargewichts so zu verfahren, dass Sedimentation und Diffusion in einem vernünftigen Verhältnis zueinander stehen. Optimale Bedingungen sind dabei besonders im Hinblick auf mögliche Fehler im Falle polydisperser Systeme dadurch definiert, dass die Verbreiterung der Gradientenkurve durch Diffusion gross sein muss gegen die Verbreiterung durch Polydispersitätseinflüsse, sodass der Polydispersitätseffekt gegen den Diffusionseffekt vernachlässigt werden kann.

Die Einstellung der stationären Geschwindigkeit ohne Auftreten störender Bremsvorgänge gelingt in einfacher Weise so, dass man kurz vor Erreichen der Endgeschwindigkeit auf den Erfahrungswert der Spannung einstellt und dann durch kurzen Druck auf das "Beschleunigungs-Relais" am Getriebekasten verhindert, dass die Endgeschwindigkeit überschritten wird. Regulierung auf annähernd zeitgleiche Signale des Antriebsstromes führen dann zur Konstanz der Umdrehungsfrequenz in den vom Getriebe bedingten engen Grenzen, ohne dass Schaltvorgänge auftreten.

3. Die Präzession des Rotors, die sich bei Ausnutzung der grossen Unwucht-Toleranz vor allem bei kleinen Winkelgeschwindigkeiten in der Abbildung des Gradienten störend bemerkbar machen kann, lässt sich—soweit sie nicht durch die

Unwucht des Rotors selbst bedingt ist—durch Tarierung von Zelle und Gegengewicht auf < 20 mg weitgehend herabmindern. (Selbstverständlich lässt sich auf diesem Wege nur die statische Unwucht ausschliessen.)

Schwieriger als die zuvor angegebenen Möglichkeiten ist das Problem der exakten Bestimmung des PHILPOT-Winkels φ und in Verbindung damit die Angabe der genauen optischen Konstanten der Ultrazentrifuge, die sich insbesondere deshalb als notwendig erweisen, weil im Interesse scharf definierter Maxima und Basislinien, sowie möglichst horizontaler Lösungsmittel-Referenzen, meist im Bereich zwischen 70 und 90°, d.h. im Bereich maximaler Empfindlichkeit der Tangensfunktion gemessen wird[*].

Eine Möglichkeit, den PHILPOT-Winkel und die optischen Konstanten (mit Ausnahme der Radialvergrösserung, die durch die Referenzabstände mit genügender Genauigkeit definiert ist) zu eliminieren, ist gegeben, wenn man am Ort der Zellmitte einen bekannten konstanten Gradienten δ in den Lichtweg der Ultrazentrifuge bringt, der auf der Photoplatte ein um den Betrag

$$\tilde{Z} = \delta/F$$

parallelverschobenes Bild des Diaphragmas liefert, wobei in

$$F = \mathrm{tg}\varphi/A \cdot f_y$$

der PHILPOT-Winkel φ, sowie der optische Hebelarm A und die Vergrösserung der Gradientenordinate am Ort des Diaphragmas durch Kamera- und Zylinderlinse f_y enthalten sind. Gelingt es also δ und $\tilde{Z}$ mit hinreichender Genauigkeit zu bestimmen, so folgt F als Quotient dieser beiden Messgrössen.

Nun steht in den prismatischen Fenstern (wedge windows) der Ultrazentrifuge ein geeigneter konstanter Gradient zur Verfügung, dessen Betrag goniometrisch oder optisch kathetometrisch ohne Schwierigkeit bestimmt werden kann[**]. Bringt man ihn durch Vertauschen der Reihenfolge von Mittelstück und Fenster mit Hilfe eines schmalen Distanzringes in Zellmitte und setzt die gesamte Anordnung zusammen mit einer leeren Normalzelle in den Rotor ein, so erhält man bei langsamer Rotation ($< 4{,}000$ Umdreh./min) zwei parallele Diaphragmabilder, deren $1/\mathrm{tg}\,\varphi$ proportionaler Abstand $\tilde{Z}$ photographisch ermittelt werden kann. (Die notwendige Tarierung setzt dabei die Verwendung von Mittelstücken etwa gleicher Masse—Kel-F, Filled Epon—voraus). Etwaige Fehler beim Zusammenfügen oder Einsetzen der Zellen wirken sich nur wenig aus; sie liegen unter $5^0/_{00}$ in $\tilde{Z}$. Wesentlich kleiner kann der Fehler bei der Bestimmung von δ gehalten werden. Selbst für die kathetometrische Bestimmung liegt er im ungünstigsten Falle bei $2^0/_{00}$. Für das von uns verwendete prismatische Fenster ergab sich bei einem Abstand von $l = 63.38 \pm 0.05$ cm eine Ablenkung $a = 0.5429 \pm 0.0009$ cm und damit $\delta_{\mathrm{rad}} = 8.5666 \cdot 10^{-3}$. Daraus folgt, wenn man auf eine Zellhöhe von $d = 1$ cm normiert bei Verwendung eines 1.2 cm-Mittelstücks

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[*] Die Genauigkeit der Angabe von φ kann bei der Justage der Optik kaum über $\pm 10'$ hinausgetrieben werden. Das entspricht in dem hier betrachteten Winkelbereich Abweichungen in D resp. s/D in der Grössenordnung von 10%.

[**] Zur kathetometrischen Bestimmung, die auf eine Messung der Verschiebung a des Bildes einer sekundären Lichtquelle (Lochblende) bei Einbringen des prismatischen Fensters in den Lichtweg und auf die Messung des Abstandes l von Fenster und Bild der Lichtquelle hinausläuft, ist es zweckmässig, durch 180°-Drehung des Fensters um seine Achse die maximale Ablenkung $2a$ nach oben und unten zu bestimmen, da auf diese Weise mögliche Fehler der Normalität von Lichtstrahl und Prismafläche reduziert werden. Strichgitter und Monochromatfilter hinter der Abbildungslinse verbessern die Messmöglichkeit durch das Auftreten von sich überschneidenden Serienbildern der Lochblende.

$$\tilde{\delta} = \frac{\delta_{\mathrm{rad}}}{d} = (7.139 \pm 0.009) \times 10^{-3}$$

In der Konstante F erscheint damit auch die Dicke der Zelle d mit einbezogen

$$F_d = \frac{F}{d} = \frac{\mathrm{tg}\varphi}{d \cdot A \cdot f_y}$$

sodass sich der in

$$f_{ji} = \frac{g_{ji}\,(\Delta n_0)^2}{2\,\pi H^2} = \frac{2\,g_{ji}}{4\pi\left[\dfrac{H}{(\Delta n_0)}\right]^2} = \frac{2\,g_{ji}}{4\pi\left[\dfrac{F_d \cdot Z_{\mathrm{max}}}{(\Delta n_0)}\right]^2} \tag{6a}$$

enthaltene konstante Anteil K ergibt zu

$$K = \frac{4\pi F_d^2}{(\Delta n_0)^2} = \frac{4\pi \mathrm{tg}^2\varphi}{(d \cdot A \cdot f_y \cdot \Delta n_0)^2} \tag{11}$$

Praktisch geht man also zweckmässig so vor, dass man dem eigentlichen Ultrazentrifugen-Versuch bei unverändertem PHILPOT-Winkel die photographische Aufnahme der Ablenkung $\tilde{Z}$ folgen lässt, aus deren Betrag in Verbindung mit $\tilde{\delta}$ die Konstante F_d mit einem Fehler von < 5‰ folgt.

Auswertung

Zur Auswertung der Aufnahmen diente ein Leitz-Tischprojektor TP 200 bzw. 20-fach vergrösserte photographische Abzüge der Platten.

Prinzipiell ergeben sich auf Grund der unterschiedlichen Beugungserscheinungen an der Phasengrenze gewisse Unterschiede in der Ausmessung der Gradienten Z, je nachdem die Phasenkante, die Phasenplatte oder ein dünner Draht als Diaphragma dienen (Fig. 1 und 2); doch kann generell für die vorliegende Methode die "Mittelpunkts-Kurve", die mit dem Normalenlineal leicht exakt konstruiert werden kann

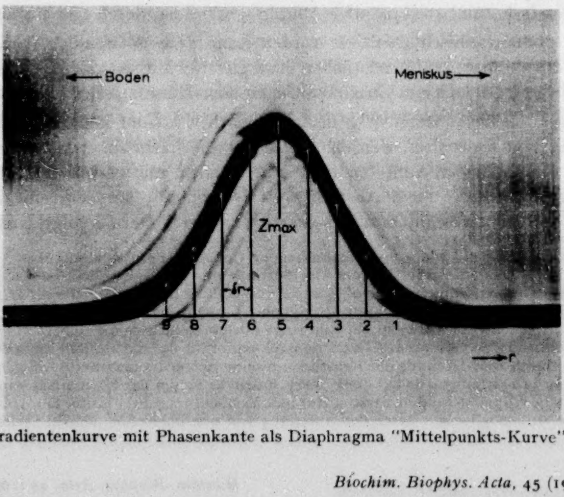

Fig. 1. Gradientenkurve mit Phasenkante als Diaphragma "Mittelpunkts-Kurve", $\varphi = 80°$.

(vgl. Fig. 1), als hinreichende Approximation an die theoretische Gradientenkurve angesehen werden. Für Phasenplatte und Draht erweist sich allerdings die das Diaphragmabild innen begrenzende "Tangenten-Kurve" (Fig. 2) als zur Auswertung besser geeignet, da sie sich zumindest auf dem photographischen Abzug als Schwarz-Weiss-Grenze leichter ausmessen lässt als die innerhalb der schwarzen Zone liegende "Mittelpunkts-Kurve", sodass in diesem Falle eine höhere Genauigkeit der Vermessung und damit eine Verminderung des Fehlers von *s* und *D* bzw. *s*/*D* erreicht werden kann. (Vgl. die Fehlerangeben in Tabelle II, Seite 228/229). Sie stellt überdies für die beiden zuletzt erwähnten optischen Systeme eine wesentlich bessere Approximation bzgl. der theoretischen Kurve im Gradientenbild dar als die "Mittelpunkts-Kurve", während für den Fall der Phasenkante die "Mittelpunkts-Kurve" als der theoretischen Kurve sehr nahekommend angesehen werden kann.

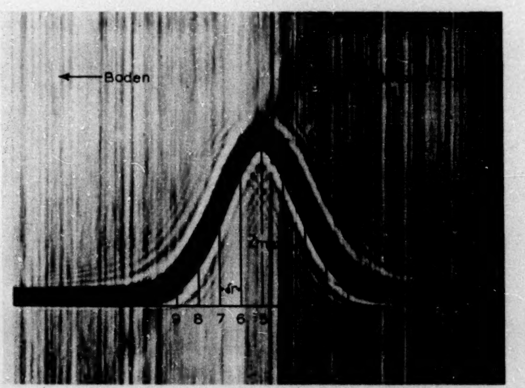

Fig. 2. Gradientenkurve mit Draht 0.08 mm Ø als Diaphragma "Tangenten-Kurve" $\varphi = 80°$.

Ermittelt man nämlich unter Zuhilfenahme zuverlässiger *s*- und *D*-Werte mit der Methode des maximalen Gradienten[2,3] die theoretischen $Z_{\max}$ und vergleicht sie mit den experimentellen Werten, so resultiert folgendes Schema, das zugleich einen Hinweis für die Wahl der richtigen Kurve bei der Anwendung der Maximalgradienten-Methode bietet:

Diaphragma	*"Tangenten-Kurve"*	*"Mittelpunkts-Kurve"*
Draht 0.08 mm Ø	Gute Übereinstimmung	Annähernde Übereinstimmung
Phasenplatte	Gute Übereinstimmung	Keine Übereinstimmung
Phasenkante	Keine Übereinstimmung (nur bedingt konstruierbar)	Sehr gute Übereinstimmung

Eine auf Boden- oder Meniskus-Referenz senkrechte Radiuslinie wird zunächst in eine beliebige Anzahl (5 bis 15) äquidistante Zonen δr definierten Abstandes r vom Rotationszentrum geteilt und die zugeordneten Höhen Z mit grösstmöglicher Genauigkeit ausgemessen. Die Basislinie berücksichtigt dabei die Schräge der Lösungsmittel-Referenz in der Weise, dass sie—parallel der zuvor erwähnten Radiuslinie—

das Mittel darstellt zwischen der Höhendifferenz der Asymptoten der Gradientenkurve*.

Bei den vorliegenden optischen Hilfsmitteln scheinen die horizontale Basis, sowie die Exaktheit der Bestimmung der Höhen wesentlichste Voraussetzungen für die Genauigkeit der Auswertungsergebnisse zu sein. Sie vermindern den Gang in den aus einer Gradientenkurve resultierenden Sedimentationskoeffizienten, der als Folge einer falschen Basislinie anzusehen ist und reduzieren ihre Streuung, die auf Ungenauigkeiten bei der Bestimmung von r und Z zurückgeht. Dabei ist von zusätzlicher Bedeutung, dass hier—im Gegensatz zur wesentlich störungsempfindlicheren 3PM[4,5]—das Maximum der Gradientenkurve, dessen Betrag ohnehin zur Bestimmung der Konstanten L (s.u.) ermittelt werden muss, zweckmässig als mittlerer r-Wert gewählt wird, da das Produkt $r^{\frac{1}{2}} \cdot Z = w$ bei der Bildung des Ausdrucks $\Delta \log w_{n}$ im Falle nahezu gleicher Z für r und $r + \delta r$ (Zonenabstände vom Maximum $= X = \pm \frac{\delta r}{2}; \pm \frac{3\delta r}{2}; \pm \frac{5\delta r}{2} \ldots$) Messfehler im Bereich des Maximums wesentlich verstärkt. (Vgl. Tab. II, S. 228/229).

Beispiele

Die numerische Auswertung sei im folgenden an Hand eines der Systeme, an denen die vorliegende Methode geprüft wurde, paradigmatisch erläutert.

Die Versuche wurden in einer Spinco-Ultrazentrifuge Modell E mit Philpot-Svensson-Optik ausgeführt. Dabei wurde angewandt: Filled-Epon Überschichtungszelle (Kapillartyp) 3°, $d = 12$ mm. Die erste Aufnahme wurde etwa 15 bis 25 Min nach Einstellung der Endgeschwindigkeit gemacht; Intervall 4′; Belichtung 20″; Versuchsdauer *ca.* 90 Min. Als Diaphragma diente (a) die Phasenkante von Spinco**; (b) eine Phasenplatte (Möller, Wedel/Holstein) und (c) ein dünner Draht von 0.08 mm Durchmesser. Die Brechungsindex-Differenzen wurden in einem Differential-Refraktometer analog dem von Brice-Phoenix gemessen; die partiellen spezifischen Volumina V_p pyknometrisch und mit Hilfe der "magnetic-float-method"[6]. Die notwendigen Konzentrationsbestimmungen wurden durch Trockenwägung bis zur Gewichtskonstanz bei 106° C vorgenommen.

Als Standard-Systeme fanden nach jeweils 24 bis 40 stündiger Dialyse folgende Substanzen verwendung (Temperatur, 20°C):

1. α-Chymotrypsinogen: "crystallized saltfree", Freehold, New Jersey. CG 450-1. IP 9.45.

~0.8 % in Natriumborat–NaOH-Puffer. pH, 9.45; μ, 0.3.

$(\Delta n_0)_{20°} = 1.5129 \cdot 10^{-3}$.

$V_{p_{20°}} = 0.722 \pm 0.004$.

2. Serumalbumin vom Rind: "trocken reinst", Behring-Werke, Marburg/L., Op. Nr. 23256. IP 4.4.

2a. ~0.8 % in Citronensäure–Dinatriumhydrogenphosphat-Puffer. pH, 4.4; μ, 0.2.

$(\Delta n_0)_{20°} = 1.6065 \cdot 10^{-3}$.

2b. ~1.0 % in Phosphat-Puffer nach SØRENSEN. pH, 7.0; μ, 0.1.

$(\Delta n_0)_{20°} = 1.7523 \cdot 10^{-3}$.

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* Diese Aussage kann nur für den hier angewandten Winkelbereich $70 < \varphi < 90$ gemacht werden.

** Fa. Beckman Instr. GmbH, München, sei auch an dieser Stelle für die leihweise Überlassung der Phasenkante gedankt.

2c. ~ 0.6 % in Acetat-Puffer nach KEGELES[7]. pH, 4.4; 0.9 % NaCl.
$(\Delta n_0)_{20°} = 1.5434 \cdot 10^{-3}$.
$V_{p20°} = 0.729 \pm 0.005$.

3. γ-Globulin vom Rind: "trocken reinst", Behring-Werke, Marburg/L., Op. Nr. 19854. IP 7.0.
~ 0.7 % in Phosphat-Puffer nach SØRENSEN. pH, 7.0; μ, 0.1.
$(\Delta n_0)_{20°} = 1.7985 \cdot 10^{-3}$.
$V_{p20°} = 0.741 \pm 0.005$.

Dem nun folgenden Rechenschema (Tabelle I) liegt Gl. (8) und (10) zugrunde. Es bedarf nur bzgl. der Konstanten näherer Erläuterung. Im übrigen kann es genügen, die Bedeutung der einzelnen Rubriken zu definieren (vgl. auch Fig. 1 und 2).

Spalte 1 gibt die Reihenfolge der δr-Zonen an in der Anordnung wachsenden Abstandes vom Rotationszentrum; Spalte 2, die auf die Zelldimension reduzierten Abstände Δr der Zonen von Meniskus- resp. Bodenreferenz in cm; Spalte 3, die Abstände vom Rotationszentrum in cm: $r = 7.3 - \Delta r$ (Bodenreferenz), $r = 5.7 + \Delta r$ (Meniskusreferenz); Spalte 4, deren Quadratwurzel auf 5 Stellen; Spalte 5, $\bar{r}_{ji} = \frac{r_j + r_i}{2}$; Spalte 6, $(\bar{r}_{ji} - r_0) \times 10^2$, mit r_0 als dem Ort des Gradienten zum Zeitpunkt t_0, der—geringe Kompressibilität des Lösungsmittels vorausgesetzt—aus dem Photogramm des Meniskus vor der Überschichtung bei niedriger Umdrehungszahl, oder generell aus dem log r/t-Diagramm[8] durch Extrapolation auf die Zeit Null folgt*; Spalte 7: $5r_0 - 4\bar{r}_{ji}$; Spalte 8, die den Zonen r zugeordneten Gradientenordinaten Z in mm, da auch $\tilde{Z}$ in mm angegeben wird (vgl. S. 221); Spalte 9, $w = r^{\frac{1}{2}} \cdot Z$; Spalte 10, log w mit fünfstelligen Logarithmen; Spalte 11, $\Delta \log w_{ji} = \log w_j - \log w_i$ unter Berücksichtigung des Vorzeichens; Spalte 12, $f_{ji} = L \cdot \Delta \log w_{ji}$. Nach Gl. (3), (6) und (6a) ist

$$f_{ji} = \frac{\Delta \ln w_{ji}}{\delta r} \cdot \frac{(\Delta n_0)^2}{2\pi H^2} = \frac{2.3026 \cdot \Delta \log w_{ji}}{\delta r} \cdot \frac{2}{\frac{4\pi F_d^2}{(\Delta n_0)^2} \cdot Z^2_{\max}}$$

Daraus ergibt sich mit (11):

$$f_{ji} = \Delta \log w_{ji} \cdot \frac{4.6052}{\delta r \cdot K \cdot Z^2{}_{\max}},$$

wobei sich der Bruch zu einer neuen Konstante

$$L = \frac{4.6052}{\delta r \cdot K \cdot Z^2{}_{\max}}$$

zusammenfassen lässt. δr auf dem photographischen Abzug oder der Platte ist darin durch Berücksichtigung der Radialvergrösserung auf Zelldimensionen zu reduzieren: z.B. auf 20 × Vergrösserung (Referenzenabstand = 32 cm) $\delta r = 0.5$ cm; daraus

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* Als Anfangszeit t_0 wurde der Zeitpunkt der Überschichtung gewählt, mit dem die Diffusion einsetzt. Diese Grösse ist bzgl. der Sedimentation jedenfalls für kleine t korrekturbedürftig, indem die Angabe der "wahren" Zeit[9] bei maximaler Beschleunigung (14 A) und den hier angewandten $\omega^2 < 2 \cdot 10^7$ zu Abweichungen in t von ≤ 3 min, d.h. in r_0 von maximal $2 \cdot 10^{-3}$ cm führt. Da sich diese Diskrepanz aber bei der numerischen Berechnung von s nach der vorliegenden Methode im Mittel nur unbedeutend äussert, während die Bestimmung von D durch sie verhältnismässig stark beeinflusst wird, kann die obige Festlegung von t_0 als gerechtfertigter Kompromiss angesehen werden.

TABELLE

RECHENSCHEMA ZUR BESTIMMUNG VON *s*, *D*

Rinderserumalbumin (2a) 0.08 mm

U = 29.500 Umdreh./min; $\omega^2 = 9.542 \cdot 10^6\ \text{sec}^{-1}$; $\varphi \simeq 82.5°$;

Aufnahme 5: $t = 2.006 \cdot 10^3$ sec; $\delta r_{\text{Zelle}} = 2.5 \cdot 10^{-2}$ cm;

1 Nr.	2 Δr (Boden)	3 r	4 $r^{1/2}$	5 $\bar{r}$	6 $(\bar{r} - r_0) \times 10^2$	7 $5r_0'' - 4\bar{r}$	8 Z	9 w	10 $\log w$	11 $\Delta \log w$
1	0.87325	6.42675	2.5351				10.83	27.455	1.43862	
				6.43925	— 1.085	6.4935				+0.28905
2	0.84825	6.45175	2.5400				21.03	53.416	1.72767	
				6.46425	+ 1.415	6.3935				+0.19666
3	0.82325	6.47675	2.5450				33.01	84.010	1.92433	
				6.48925	+ 3.915	6.2935				+0.09950
4	0.79825	6.50175	2.5499				41.43	105.642	1.02383	
				6.51425	+ 6.415	6.1935				— 0.08652
5	0.77325	6.52675	2.5548				33.88	86.557	1.93731	
				6.53925	+ 8.915	6.0935				— 0.20015
6	0.74825	6.55175	2.5596				21.33	54.596	1.73716	
				6.56425	+11.415	5.9935				— 0.31831
7	0.72325	6.57675	2.5643				10.23	26.233	1.41885	

Aufnahme 16: $t = 4.648 \cdot 10^3$ sec; $\delta r_{\text{Zelle}} = 2.5 \cdot 10^{-2}$ cm;

1 Nr.	2 Δr (Boden)	3 r	4 $r^{1/2}$	5 $\bar{r}$	6 $(\bar{r} - r_0) \times 10^2$	7 $5r_0'' - 4\bar{r}$	8 Z	9 w	10 $\log w$	11 $\Delta \log w$
1	0.83625	6.46375	2.5424				7.75	19.704	1.29456	
				6.47625	+ 2.615	6.3455				+0.20842
2	0.81125	6.48875	2.5473				12.50	31.841	1.50298	
				6.50125	+ 5.115	6.2455				+0.15435
3	0.78625	6.51375	2.5522				17.80	45.429	1.65733	
				6.52625	+ 7.615	6.1455				+0.11120
4	0.76125	6.53875	2.5571				22.95	58.685	1.76853	
				6.55125	+10.115	6.0455				+0.04914
5	0.73625	6.56375	2.5620				25.65	65.715	1.81767	
				6.57625	+12.615	5.9455				—0.02356
6	0.71125	6.58875	2.5668				24.25	62.245	1.79411	
				6.60125	+15.115	5.8455				—0.09052
7	0.68625	6.61375	2.5717				19.65	50.534	1.70359	
				6.62625	+17.615	5.7455				—0.13418
8	0.66125	6.63875	2.5766				14.40	37.103	1.56941	
				6.65125	+20.115	5.6455				—0.20090
9	0.63625	6.66375	2.5814				9.05	23.362	1.36851	

$\delta r_{\text{Zelle}} = \frac{0.5}{20} = 2.5 \cdot 10^{-2}\,cm$; Spalte 13, die Summe von Spalte 6 und 12 unter Berücksichtigung des Vorzeichens; Spalte 14, βt als Quotient aus Spalte 13 und 7, entsprechend Gl. (8); Spalte 15, der Sedimentationskoeffizient aus Spalte 14 gemäss $s = \beta t/\omega^2 t$; Spalte 19: das Produkt $(N \cdot \Delta \log w_{jt}) \times 10^{-5}$ mit $N = \frac{4.6052 \cdot t}{\delta r}$, das als Nenner in Gl. (10) resultiert, wenn man g_{jt} durch $\frac{2.3026 \cdot \Delta \log w_{jt}}{\delta r}$ ersetzt (Gl. (3)).

UND *s*/*D* NACH DER ZWEIPUNKT-METHODE

Draht-Diaphragma Versuch Nr. 1

$\tilde{Z} = 22.01$ mm; $F = 3.2435 \cdot 10^{-4}$; $K = 5.1199 \cdot 10^{-1}$;
$Z_{max} = 41.43$ mm; $L = 2.0961 \cdot 10^{-1}$; $N = 3.6952 \cdot 10^{5}$.

12 $f_{ji} \times 10^2$	13 $f_{ji} + (\bar{r} - r_0) \times 10^2$	14 $\beta t \times 10^2$	15 $s \times 10^{13}$	16 $1 - \beta t$	17 $\bar{r}(1 - \beta t)$	18 $r_0 - \bar{r}(1 - \beta t) \times 10^2$	19 $N\Delta \log w \times 10^{-5}$	20 $D \times 10^7$	21 $s/D \times 10^7$
+6.05884	4.974	0.7660	4.00	0.99234	6.38993	+ 6.02	+1.06810	5.64	7.09
+4.12223	5.537	0.8661	4.52	0.99134	6.40826	+ 4.18	+0.72670	5.75	7.86
+2.08564	6.001	0.9535	4.98	0.99047	6.42738	+ 2.27	+0.36767	6.17	8.07
−1.81356	4.601	0.7429	3.88	0.99257	6.46586	− 1.58	−0.31971	4.94	7.85
−4.19539	4.720	0.7746	4.05	0.99225	6.48860	− 3.85	−0.73960	5.21	7.77
−6.67216	4.743	0.7913	4.13	0.99209	6.51231	− 6.22	−1.17622	5.29	7.81

$<s> = 4.26 \cdot 10^{-13}$ $<D> = 5.50 \cdot 10^{-7}$ $<s/D> = 7.74 \cdot 10^{-7}$

$Z_{max} = 25.65$ mm; $L = 5{,}4768 \cdot 10^{-1}$; $N = 8.5620 \cdot 10^{5}$.

12 $f_{ji} \times 10^2$	13 $f_{ji} + (\bar{r} - r_0) \times 10^2$	14 $\beta t \times 10^2$	15 $s \times 10^{13}$	16 $1 - \beta t$	17 $\bar{r}(1 - \beta t)$	18 $r_0 - \bar{r}(1 - \beta t) \times 10^2$	19 $N\Delta \log w \times 10^{-5}$	20 $D \times 10^7$	21 $s/D \times 10^7$
+11.4147	14.030	2.2110	4.98	0.97789	6.3330	+11.71	+1.7845	6.56	7.59
+ 8.4534	13.568	2.1724	4.90	0.97827	6.3600	+ 9.01	+1.3215	6.82	7.18
+ 6.0902	13.705	2.2301	5.03	0.97770	6.3807	+ 6.45	+0.9521	6.77	7.43
+ 2.6913	12.806	2.1183	4.78	0.97882	6.4125	+ 3.76	+0.4207	8.95	5.34
− 1.2903	11.325	1.9048	4.29	0.98095	6.4510	− 0.09	−0.2017	4.46	9.62
− 4.9576	10.157	1.7376	3.92	0.98262	6.4865	− 3.64	−0.7750	4.70	8.34
− 7.3488	10.266	1.7868	4.03	0.98213	6.5079	− 5.78	−1.1489	5.03	8.01
−11.0029	9.112	1.6150	3.64	0.98385	6.5438	− 9.37	−1.7201	5.45	6.68

$<s> = 4.45 \cdot 10^{-13}$ $<D> = 6.09 \cdot 10^{-7}$ $<s/D> = 7.52 \cdot 10^{-7}$

MESSERGEBNISSE UND DISKUSSION

Die Ergebnisse der Messungen mit den Angaben des mittleren Fehlers der für eine Kurve aus den einzelnen Zonen gemittelten <*s*>-, <*D*>- und <*s*/*D*>-Werte, sowie der Streuung der für jeweils drei Gradientenkurven gegen Anfang, Mitte und Ende eines Versuches gewonnenen Mittelwerte «*s*», «*D*» und «*s*/*D*» finden sich, zusammen mit den aus SM bzw. MGM ermittelten s_s bzw. D_z und den ergänzenden Angaben des

TABELLE

ZUSAMMENSTELLUNG

Versuch Nr.	*Substanz, Diaphragma*	*φ°*	*t_{sec}*	*U*	$«s»_{20^\circ} \times 10^{13}$	
1	Serumalbumin 2a	82.5	2006	29,500	4.26 ± 0.38	8.88 %
			2966		4.30 ± 0.53	12.38
	Draht 0.08 mm		4648		4.45 ± 0.51	11.42
					«s» = 4.34 ± 0.06	1.33 %
					s_s = 4.16 ± 0.05	1.25 %
			2006		4.26 ± 0.19	4.43 %
			2966		4.35 ± 0.17	3.97
			4648		4.41 ± 0.13	2.94
					«s» = 4.34 ± 0.04	1.03 %
			2006		4.32 ± 0.86	20.00 %
			4648		4.22 ± 0.40	9.48
					«s» = 4.27 ± 0.05	1.17 %
2	Serumalbumin 2a	85	1318	29,500	4.04 ± 0.88	21.75 %
			2998		4.17 ± 0.14	3.35
	Phasenplatte		3959		4.19 ± 0.43	10.33
					«s» = 4.13 ± 0.05	1.14 %
					s_s = 4.10 ± 0.05	1.20 %
3	Serumalbumin 2a	87.5	1650	29,500	4.04 ± 0.29	7.28 %
			3090		4.29 ± 0.24	5.55 %
	Draht 0.08 mm		4531		4.47 ± 0.31	7.00
					«s» = 4.26 ± 0.12	2.93 %
					s_s = 4.05 ± 0.08	2.0 %
			1650		4.04 ± 0.24	5.88 %
			3090		4.25 ± 0.23	5.40
			4531		4.51 ± 0.30	6.63
					«s» = 4.27 ± 0.14	3.19 %
4	Serumalbumin 2c	80	2239	29,500	3.89 ± 0.68	17.45 %
			3690		4.56 ± 0.39	8.54
	Phasenkante				«s» = 4.23 ± 0.34	8.00 %
					s_s = 4.26 ± 0.39	9.15 %
5	Serumalbumin 2b	85	1513	29,500	3.76 ± 1.08	28.63 %
			3435		4.63 ± 0.44	9.42
	Draht 0.08 mm		5835		4.98 ± 0.28	5.54
					«s» = 4.46 ± 0.36	8.13 %
					s_s = 4.42 ± 0.08	1.81 %
6	α-Chymotrypsinogen 1	85	1144	50,740	2.26 ± 0.46	20.38 %
			2826		2.57 ± 0.26	10.10
	Draht 0.08 mm		4507		2.96 ± 0.29	9.92
					«s» = 2.60 ± 0.20	7.79 %
					s_s = 2.48 ± 0.12	4.83 %
7	γ-Globulin 3	85	2305	16,200	6.47 ± 2.81	43.46 %
			3746		6.83 ± 1.79	26.19
	Draht 0.08 mm		5188		7.09 ± 1.55	21.89
					«s» = 6.79 ± 0.18	2.67 %
					s_s = 7.06 ± 0.18	2.56 %

II

DER MESSERGEBNISSE

$\langle D\rangle_{20^\circ} \times 10^7$		$\langle s/D\rangle \times 10^7$		*Auswertungsmethode*	
5.50 ± 0.39	7.14 %	7.74 ± 0.30	3.92 %	2PM	Tangentialkurve
5.92 ± 0.75	12.62	7.28 ± 0.58	7.90		
6.09 ± 1.38	22.64	7.52 ± 1.17	15.53 %	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
«*D*» = 5.84 ± 0.18	3.00 %	«(*s*/*D*)» = 7.51 ± 0.13	1.77 %		
D_z = 5.65 ± 0.25	4.5 %			SM	MGM
5.59 ± 0.66	11.79 %	7.75 ± 1.20	15.45 %	2PM	Mittelpunktskurve
5.45 ± 1.31	24.06	8.68 ± 3.38	38.92		
5.00 ± 3.30	65.96	4.68 ± 9.12	195.0	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
«*D*» = 5.35 ± 0.18	3.33 %	«(*s*/*D*)» = 7.04 ± 1.21	17.16 %		
6.38 ± 0.67	10.48 %	6.82 ± 1.73	25.29 %	3PM	Tangentialkurve
5.20 ± 1.06	20.30	8.44 ± 1.67	19.75		
«*D*» = 5.79 ± 0.59	10.19 %	«(*s*/*D*)» = 7.63 ± 0.81	10.60 %	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
5.65 ± 0.18	3.16 %	7.13 ± 1.40	19.64 %	2PM	Tangentialkurve
5.22 ± 0.96	18.41	8.18 ± 1.35	16.50		
5.69 ± 1.66	29.24	8.36 ± 3.77	45.14	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
«*D*» = 5.52 ± 0.15	2.72 %	«(*s*/*D*)» = 7.89 ± 0.38	4.86 %		
D_z = 6.01 ± 0.48	8.0 %			SM	MGM
5.47 ± 0.28	5.12 %	7.41 ± 0.34	4.50 %	2PM	Tangentialkurve
5.00 ± 1.02	20.38	9.01 ± 1.96	21.75		
5.81 ± 1.57	27.04	8.13 ± 1.70	20.90	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
«*D*» = 5.43 ± 0.23	4.32 %	«(*s*/*D*)» = 8.18 ± 0.47	5.69 %		
D_z = 5.40 ± 0.27	5.0 %			SM	MGM
5.00 ± 0.74	14.74 %	8.28 ± 1.33	16.06 %	2PM	Tangentialkurve
7.32 ± 4.17	56.89	7.09 ± 2.59	36.50		
7.74 ± 4.60	59.44	7.39 ± 2.76	37.35	$X = \pm \delta r/2; \pm 3\delta r/2; \pm 5\delta r/2 \cdots$	
«*D*» = 6.69 ± 0.85	12.74 %	«(*s*/*D*)» = 7.59 ± 0.36	4.71 %		
6.90 ± 0.68	9.91 %	5.68 ± 1.05	18.56 %	2PM	Mittelpunktskurve
7.08 ± 0.21	30.12	7.03 ± 2.22	31.50		
«*D*» = 6.99 ± 0.09	1.27 %	«(*s*/*D*)» = 6.35 ± 0.68	10.62 %	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
D_z = 6.40 ± 0.40	6.25 %			SM	MGM
6.19 ± 1.24	19.99 %	6.56 ± 2.96	45.09 %	2PM	Tangentialkurve
6.73 ± 1.51	22.41	7.13 ± 1.25	17.52		
5.06 ± 8.99	177.6	6.72 ± 5.26	78.21	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
«*D*» = 5.99 ± 0.49	8.21 %	«(*s*/*D*)» = 6.80 ± 0.17	2.50 %		
				SM	
10.95 ± 0.71	6.50 %	2.05 ± 0.35	16.91 %	2PM	Tangentialkurve
12.00 ± 5.37	44.77	3.25 ± 3.22	99.15		
13.48 ± 16.15	119.8	1.57 ± 2.11	134.5	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
«*D*» = 12.14 ± 0.73	6.04 %	«(*s*/*D*)» = 2.29 ± 0.50	22.0 %		
				SM	
4.47 ± 0.18	3.98 %	14.28 ± 5.86	41.01 %	2PM	Tangentialkurve
4.55 ± 0.24	5.33	14.89 ± 3.40	22.86		
4.62 ± 0.64	13.92	15.54 ± 3.44	22.11	$X = 0; \pm \delta r; \pm 2\delta r; \pm 3\delta r \cdots$	
«*D*» = 4.55 ± 0.13	2.92 %	«(*s*/*D*)» = 14.90 ± 0.36	2.41 %		
				SM	

PHILPOT-Winkels, der Aufnahmezeit t, der gewählten Umdrehungszahl des Rotors U_{UpM} und des Auswertungsverfahrens in Tabelle II zusammengefasst. (Die Ziffern bei den Angaben über die Versuchssubstanzen verweisen dabei auf die Zusammenstellung der Standard-Systeme auf Seite 224/225).

Wie Tabelle II zeigt, resultiert für die verschiedenen angewandten Methoden befriedigende Übereinstimmung, die auch den Vergleich mit den in der Literatur angegebenen Daten zulässt (Tabelle III)*.

Für mittlere Molekulargewichte ergibt sich insgesamt bei Zugrundelegung optimaler Bedingungen des Experiments und der Auswertung (Draht-Diaphragma, "Tangenten-Kurve"), wenn man die Ergebnisse verschiedener Messungen am gleichen System vergleicht, eine mittlere Abweichung der «s», «D» und «s/D» von $< 2\%$, während als maximaler Fehler (etwa für den Fall Phasenkante, "Mittelpunkts-Kurve") 10 % angenommen werden müssen.

TABELLE III

VERGLEICH DER MESSERGEBNISSE MIT LITERATUR-WERTEN

Substanz	$s_{20°,w} \cdot 10^{13}$	$s_{Lit} \cdot 10^{13}$	$D_{20°,w} \cdot 10^{7}$	$D_{Lit} \cdot 10^{7}$	$M_{2PM} \cdot 10^{-3}$	$M_{Lit} \cdot 10^{-3}$
α-Chymotrypsinogen	2.60	2.5	12.14	10.4	21.4	22
Serumalbumin vom Rind	4.26	4.28	5.66	6.15	68.6	68
γ-Globulin vom Rind	6.94	7	4.65	4.1	143	(155)§

§ Berechnet aus s und D.

Es erscheint nicht angebracht, hier eine mehr ins Detail gehende Fehlerbetrachtung anzustellen, da sich bei den Berechnungen eine grosse Zahl systematischer Fehlerquellen mit den Subjektiven der Auswertung der photographischen Aufnahmen in unübersichtlicher Weise überlagert. Es sei nur angedeutet, dass ein Teil der erwähnten subjektiven Fehlerursachen einer falschen Basis bzw. ungenauer r- und Z-Bestimmungen durch Einführen von Fehlergewichten einer präzisen Fehlerberechnung zugänglich ist. Diese folgen daraus, dass maximale Genauigkeit im Bereich kleiner Steigung in der Nähe des Maximums der Gradientenkurve gewährleistet ist, während das Maximum selbst aus Gründen der Optik und die übrigen Kurventeile wegen der kleinen Absolutbeträge von Z resp. wegen der grösseren Steigung der Kurve weniger genau ausgemessen werden können. Ein diesen Sachverhalt quantitativ berücksichtigendes Verfahren zur Fehlerberechnung erweist sich allerdings für eine Routine-Methode als nicht sinnvoll und soll daher hier nicht näher diskutiert werden, zumal es die systematischen Fehler nicht einzubeziehen erlaubt, die aus dem Überschichtungsvorgang, der Feldänderung bei der Beschleunigung bis zum Erreichen der Endgeschwindigkeit und der Problematik der "wahren" Anfangszeit t_0 bzw. des exakten r_0-Wertes etc. resultieren. Auf sie wird man in erster Linie die Beobachtung zurückführen können, dass sich entgegen der mathematischen Voraussetzung bei mittleren Zeiten die zuverlässigsten s- und D-Werte ergeben, da dann der Kompromiss vorliegt zwischen den Überschichtungs- und Beschleunigungseffekten und der Bedingung der Gl. (7), die zusammen mit unvermeidlichen Störungen der Diffusion durch Unregelmässigkeiten in den äusseren Versuchsbedingungen längere Messzeiten, bei denen der Fehler der "wahren" Zeit nicht mehr ins Gewicht fiele, verbieten.

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* Bei den in Tabelle II und III zusammengefassten Daten wurde Idealität der Lösungen angenommen. Der Einfluss von Konzentration und Kreuzkoeffizienten wurde also vernachlässigt. Im Falle stark konzentrationsabhängiger monodisperser Systeme lässt sich aus einem Versuch mit Hilfe der 2PM ein Hinweis auf den Grad der Konzentrationsabhängigkeit gewinnen.—Die Literaturwerte stellen unter vergleichbaren Bedingungen gewonnene Mittelwerte dar[10-13].

Es soll im folgenden nicht versucht werden, den Einfluss von pH und Ionenstärke bei den oben aufgeführten Messungen zu interpretieren. Die diesbezüglichen orientierenden Versuche sollten nur die Grenzen erkennen lassen, wie weit die theoretische Voraussetzung nicht elektrolytischer bzw. im IP befindlicher Lösungen ernst genommen werden muss. Wie das Ergebnis zeigt, liegen die Abweichungen im Bereich der Messfehler (vgl. Versuch No. 4 mit No. 1, 2 und 3), sodass wenigstens für den Fall der hier verwendeten Ionenstärken (μ, 0.1–0.2) eine Beschränkung auf den isoelektrischen Bereich nicht als notwendig anzusehen ist*.

Fasst man nun die bisher mitgeteilten Methoden zur Bestimmung des Quotienten s/D zusammen, so ergeben sich für die vorliegenden optischen Hilfsmittel drei Möglichkeiten: (a) die Kombination der SVEDBERG-Methode (SM), die s liefert, mit der Maximalgradienten-Methode (MGM), aus der mit guter Genauigkeit D bestimmt werden kann**, (b) die Dreipunkt-Methode (3PM), aus der unmittelbar s/D resultiert und (c) die hier dargestellte Zweipunkt-Methode (2PM). Dabei ist die erste Möglichkeit mit einer gewissen Unsicherheit belastet, da die beiden zugrundeliegenden Methoden auf verschiedenen theoretischen Ableitungen beruhen; sie soll deshalb bei dem Vergleich der Methoden nicht ausführlicher betrachtet werden. Es sei nur hervorgehoben, dass die aus der SM resultierenden s_s sich im wesentlichen im Bereich der Fehlergrenzen mit den «s»-Werten der 2PM decken*** und dass bzgl. der Fehlermöglichkeiten die aus der MGM gewonnenen Diffusionskoeffizienten durch Unsicherheiten in Z empfindlich gestört werden, während im Falle der 2PM Zeitfehler stärker ins Gewicht fallen können.

Vergleicht man nun die Ergebnisse der 3PM (s. auch GEHATIA UND HÜBNER[5]) mit denen der 2PM, so ergibt sich zunächst für die bisher untersuchten molekulareinheitlichen Systeme, dass der bei der 2PM auftretende mittlere Fehler nur etwa ein Drittel bis ein Viertel des Fehlers der 3PM ausmacht. Diese Erfahrung beruht im wesentlichen darauf, dass für die 3PM eine äusserst genaue Festlegung der Basishöhe erforderlich ist, da bei ihr direkt die Z-Beträge in die Rechnung eingehen, während im Falle der 2PM die Division durch den auf die gleiche Basis bezogenen Maximalgradienten (vgl. Gl. (6)) mögliche Basisfehler stark vermindert. Ebenso werden Fehler in der Neigung der Basis, die zu einem mehr oder weniger deutlichen Gang in den für die betreffende Kurve berechneten s/D-Werten führt, von der 3PM deutlicher registriert als von der 2PM.

In dieser Tatsache liegt zugleich eine gewisse Beeinträchtigung der Anwendbarkeit der 3PM zur Polydispersitätsbestimmung, die in anderer Weise auch für die 2PM gilt. Während nämlich die 3PM—eine sehr gute Optik vorausgesetzt—durch zonenweise Bestimmung von s/D gute Hinweise auf das Vorliegen von Polydispersität ermöglicht, die eben durch die zuvor erwähnte Basis-Empfindlichkeit eingeschränkt wird, indem bei falscher Neigung auch einheitliche Systeme als polydispers erscheinen können, führt die 2PM auf Grund der Division durch $Z_{\max}$ zu s/D-Werten für die einzelnen Zonen, die nur relative Polydispersitätshinweise gestatten. Überdies führt das Beziehen auf $Z_{\max}$, das ja einem mittleren s/D äquivalent ist, zu einer Verminderung der Empfindlichkeit bei der Untersuchung der Polydispersität.

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* Eine Erklärung für die gerade bei Entfernung vom IP auftretenden grösseren Schwankungen in s und D kann sicher nicht in den elektrostatischen Wechselwirkungen zwischen den Teilchen gesucht werden.

** Der Ordinatenabschnitt der h-Funktion[2], der auch s/D direkt zu bestimmen erlaubt, kann nur als Näherung angesehen werden.

*** Die Abweichungen ($<$ 2%) sind wohl auf subjektive Fehler bei der 2PM zurückzuführen.

Zusammenfassend kann also gesagt werden, dass die vorliegende Methode in erster Linie zur Molekulargewichtsbestimmung molekulareinheitlicher Systeme mit nicht zu grosser Konzentrationsabhängigkeit von s und D herangezogen werden wird, wobei infolge der verhältnismässig geringen Empfindlichkeit gegenüber den Mängeln der optischen Analysenmethoden alle üblichen optischen Systeme angewandt werden können.

Bezüglich der Polydispersität, die mit den hier diskutierten Methoden selbstverständlich nur qualitativ angezeigt, nicht aber quantitativ bis zur Ermittlung der Verteilungsfunktion bestimmt werden kann, wird man, je nach dem Polydispersitätsgrad und der zugrundeliegenden Optik zweckmässig die 2PM oder die 3PM heranziehen, wie das folgende Schema angibt:

System	*Hilfsmittel*	*Methode*
Monodispers	Übliche optische Methoden	2PM
Paucidispers	Übliche optische Methoden	3PM
Polydispers (schwach)	Übliche optische Methoden	—
Polydispers (schwach)	Sehr gute optische Methoden (evtl. Rayleigh-Optik)	3PM
Polydispers (stark)	Übliche optische Methoden	2PM
Polydispers (stark)	Sehr gute optische Methoden (evtl. Rayleigh-Optik)	3PM

Über die so zu gewinnenden Hinweise hinaus kann die 2PM in Verbindung mit der Momenten-Methode[2] auch als Hilfsmittel zur quantitativen Polydispersitätsanalyse angesehen werden. Doch erweist sich dazu eine vervollkommnete Optik als unumgängliche Voraussetzung, da die höheren Momente—wie Versuche in dieser Richtung zeigten—mit den gebräuchlichen optischen Hilfmitteln bisher kaum mit genügender Genauigkeit bestimmt werden können.

Abschliessend bleibt noch zu erwähnen, dass die vorliegende Methode die zur Bestimmung des Molekulargewichts notwendigen Daten aus sehr kurzen Messzeiten resp. aus wenigen photographischen Aufnahmen—im Grenzfall aus einer einzigen Kurve–zu gewinnen erlaubt. Sie kann daher beispielsweise auch zur Molekulargewichtsbestimmung instabiler Systeme dienen, die sich während längerer Messzeiten in irgendeiner Weise in ihrem Sedimentations—bzw. Diffusionsverhalten verändern und erlaubt es, derartige Veränderungen in ihrem Verlauf quantitativ messend zu verfolgen.

DANK

Wir wollen nicht versäumen, Herrn Prof. Dr. J. STAUFF auch an dieser Stelle sehr herzlich für seine überaus freundliche Förderung zu danken.

Ebenso gilt unser Dank der Deutschen Forschungsgemeinschaft, die die Arbeit durch Sach- und Personalmittel grosszügig unterstützte.

ZUSAMMENFASSUNG

Es wird—basierend auf der früher dargestellten Theorie der Brownschen Bewegung im Zentrifugalfeld—eine neue Möglichkeit angegeben, mit Hilfe der gebräuchlichen optischen Systeme Sedimentations- und Diffusionskoeffizienten aus zwei Punkten und dem Maximum der experimentellen Gradientenkurve der Ultrazentrifuge zu gewinnen.

Die experimentellen Voraussetzungen und die Prüfung der Methode an Hand dreier Standard-Substanzen im Molekulargewichtsbereich zwischen 20,000 und 150,000 werden mitgeteilt. Es ergibt sich für die untersuchten Systeme gute Übereinstimmung mit den in der Literatur angegebenen Werten.

Der mittlere Fehler der Angaben von s und D beträgt für den Fall mittlerer Molekulargewichte unter optimalen Bedingungen des Experiments und der Auswertung 1 bis 3 %, d.h. nur *ca.* ein Drittel des an anderer Stelle für die 3PM mitgeteilten Wertes. Im Bereich kleiner Molekulargewichte liegt er unter 8 %. Die Schwankungen in s/D liegen für mittlere Molekulargewichte unter 5 %, für kleine um 20 %.

Durch Vergleich mit den bisher verwendeten Methoden lassen sich deren Anwendungsbereiche gegenüber der hier dargestellten 2PM abgrenzen. Danach erscheint die vorliegende Methode vorzugsweise für ideale, molekulareinheitliche Systeme geeignet oder aber für stark polydisperse, deren Uneinheitlichkeit sie qualitativ nachzuweisen erlaubt.

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PURIFICATION AND PROPERTIES OF ADRENAL ACID PROTEINASE

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SUMMARY

The proteinase activities at pH 3.5 (haemoglobin substrate) of extracts of a number of bovine tissues, including endocrine glands, have been compared. All endocrine extracts tested were highly active at this pH.

Extracts of thyroid and of adrenal glands, when tested for proteinase activity (haemoglobin substrate) over a range of pH values, showed two pH optima, one in the acid and one in the alkaline range, thus resembling extracts of pituitary glands[1].

A proteinase optimally active between pH 3.5 and pH 4.0 has been purified approx. 70-fold from extracts of bovine adrenal glands and some of its properties are reported. The enzyme had no action on any of the synthetic peptide substrates tested.

INTRODUCTION

Studies of the proteolytic systems of the endocrine glands have been reported for the pituitary by ADAMS AND SMITH[1] and for the thyroid by MCQUILLAN AND TRIKOJUS[2] and LAVER AND TRIKOJUS[3,4]. Extracts of both glands exhibited proteinase activity optimal between pH 3 and pH 4 and, in addition, pituitary extracts showed a second peak of activity in the alkaline range. In the present investigations a comparison of the acid proteinase content of extracts of beef endocrine glands, muscle and liver revealed a relatively high activity in adrenal tissue. Extracts of adrenal and of thyroid glands, when tested against haemoglobin over a range of pH values, showed the same pattern of activity as found by ADAMS AND SMITH[1] for anterior pituitary extracts. The enzyme active at acid pH, present in adrenal glands, has been purified approx. 70-fold and some of its properties have been studied.

MATERIALS AND METHODS

Bovine glands, liver and muscle were obtained fresh frozen from the abattoirs, and dissected free from fat and connective tissue.

Chemicals used were of A.R. quality. Acetone was freshly distilled over NaOH before use.

Proteolytic activity was assayed by the method of ANSON[5], with minor modifications. One unit of proteinase activity refers to the amount of enzyme required to liberate 10^{-4} mequiv. tyrosine in 30 min at 37° with haemoglobin as substrate (pH 3.5).

For the determination of the pH-activity curves of the purified proteinase and of saline extracts of adrenal and of thyroid glands, urea-denatured haemoglobin and KUNITZ's[6] modification of ANSON's method was used.

Protein-nitrogen was determined by the micro-Kjeldahl procedure, with selenium as catalyst. Before digestion, samples were submitted to prolonged dialysis at 2°.

Paper chromatography was carried out using No. 1 Whatman filter paper at constant temperature (25°) with butanol–acetic acid–water (4:1:1) as the descending phase. (For benzoyl-L-arginine amide, L-leucine amide and glycyl-L-proline the descending phase was *n*-propanol–water (4:1)). Samples (5 µl) were applied to the paper in a single application using a platinum loop. The chromatograms were allowed to run for 8 or 16 h, then dried and developed with ninhydrin. (Chromatograms involving benzoyl-L-arginine amide were developed using SAKAGUCHI's reagents.)

EXPERIMENTAL

Proteinase activity of beef tissue extracts

The extracts were made by blending the tissue with 0.9% saline (3 ml/g) for 1 min in a Waring blendor at 2°. After the addition of toluol (0.1 ml/100 ml extract) the mixtures were allowed to stand overnight in the cold room and then centrifuged at 3,000 × *g* for 2 h. The supernatants were filtered through cotton wool and dialysed at 2° for 48 h against four changes of distilled water. Duplicate nitrogen and proteinase determinations were carried out on these solutions.

The results are set out in Table I and show that all the extracts tested were active at pH 3.5. The number of units/g of tissue is much lower in muscle than in the other extracts. The concentration of activity in the extracted protein (units/mg N) is highest in extracts of adrenal and ovary.

The proteinase activity of saline extracts of adrenal and thyroid glands over a range of pH values is shown in Fig. 1. For comparison, the curve obtained from extracts of anterior pituitary glands by ADAMS AND SMITH[1] is included. In each case there are two distinct peaks of activity—one between pH 3.5 and pH 4.0, the other between pH 8.5 and pH 9.5.

TABLE I

COMPARISON OF PROTEINASE ACTIVITY OF DIALYSED SALINE EXTRACTS OF BEEF TISSUE

Substrate: acid-denatured haemoglobin. Incubation: 30 min, 37°, pH 3.5.

	Muscle	*Pituitary*		*Thyroid*	*Adrenal*	*Ovary*		*Liver*
		Anterior	*Posterior*			*Total*	*(Corp. lut.)*	
Units*/g tissue wet wt.	22	270	230	300	320	355	(85)	500
Units/mg N in extract	6	41	33	16	57	77	(18)	41
N (mg) extracted/g tissue wet wt.	3.8	6.5	6.9	19	5.6	4.6	(4.6)	12.3

* For definition see MATERIALS AND METHODS.

Purification of the adrenal proteinase optimally active between pH 3.5 and pH 4.0

Initial experiments followed the method adopted by LAVER AND TRIKOJUS[3] for thyroid proteinase in which the bulk of contaminating protein is removed by precipitation at pH 3.5, further purification being effected by fractionation with acetone. However, with adrenal extracts the final products were only about 0.1 as active as those from thyroid tissue. By including a step involving incubation of the extracts at a pH value within the optimal range of the adrenal enzyme, potency was increased without reduction in the total enzymic activity. Material so treated was also much more susceptible to further purification by acetone fractionation. The procedure adopted was as follows (see also Table II):

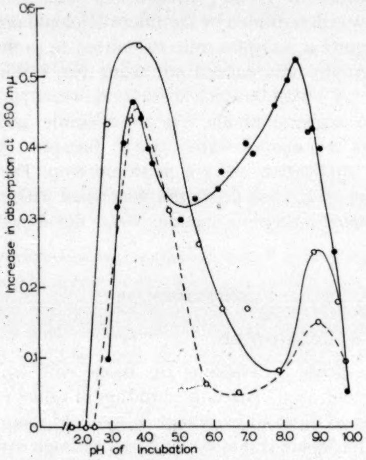

Fig. 1. Variation with pH in proteinase activity of saline extracts of bovine thyroid and adrenal glands. The curve obtained by ADAMS AND SMITH[1] with extracts of hog anterior pituitary glands is included for comparison. Substrate: bovine haemoglobin. Incubation: 30 min, 37°. ○—○, adrenal; ○- - -○, thyroid; ●—●, pituitary (ADAMS AND SMITH[1]).

Step 1

The dissected glands (1,000 g) were blended at 0° with saline (0.9%; 3,000 ml) for 1 min in a Waring blendor, toluol (1 ml) was added and the suspension transferred to the cold room overnight (saline extract; Table II). Cold acetone (1,000 ml) was then added slowly with stirring and the mixture centrifuged at 3,000 × *g* (1 h). The addition of acetone increased the number of units extracted (probably due to the removal of fatty material) and it also facilitated centrifugation. (This was also found to be the case in extracting thyroid tissue for acid proteinase). The residue was re-blended for 0.25 min with cold saline (300 ml) plus acetone (to 20% v/v) and centrifuged. The supernatants were combined to form the saline–acetone extract (Table II).

Step 2

The extract was gently stirred in an ice bath and pre-cooled acetone (below

—10°) gradually added to 80 % (v/v). After 30 min the clear supernatant was siphoned off as far as possible and the remaining suspension rapidly filtered at 2° with light suction. The precipitate was washed with cold 80 % acetone (750 ml) and acetone (2 × 250 ml) and immediately transferred to water (2,000 ml) with hand stirring.

Step 3

The cold slightly turbid solution was brought to pH 4.5 with 2 *M* HCl and allowed to stand for 10 min at 2°. The insoluble material was removed by centrifuging, washed with cold water adjusted to pH 4.5 (250 ml) and discarded.

Step 4

The supernatant and washing were combined and pre-cooled acetone (below —10°) gradually added to 80 % (v/v) as in *Step 2*. After standing for 30 min, the clear supernatant was siphoned off and the remaining suspension centrifuged. The residue was dissolved in water (350 ml) and dialysed. Insoluble material was removed by centrifuging, washed once with water and then discarded.

Step 5

The clear supernatant (including washing: 1,140 ml) from *Step 4* was adjusted to pH 3.9 by the gradual addition of NaAc buffer (0.5 *M*, pH 3.6; 47.5 ml). Toluol (1 ml) was added and the solution was gently shaken in two heat-sterilized stoppered bottles at 37° for 5 h. After standing overnight at 2° the solution was dialysed. In early preparations this solution was freeze dried and then, prior to acetone fractionation, dissolved in NH_4Ac buffer (0.02 *M*, pH 5.0) to give a 1 % solution. However, in subsequent preparations it was found more convenient to avoid freeze drying and to bring the solution to 80 % (v/v) with acetone and dissolve the precipitate in water (300 ml); after removal of the acetone by dialysis the solution was brought to pH 5.0 by the addition of 0.2 times its volume of NH_4Ac buffer (0.1 *M*, pH 5.0). In this way, the recovery of enzyme during the subsequent fractionation procedure was increased.

Acetone fractionation

The buffered solution prepared above was brought successively to 40 %, 55 % and 80 % (v/v) with acetone. The precipitates at each concentration of acetone (Fractions I, II and III respectively) were collected by centrifuging, washed once with the appropriate concentration of acetone, dissolved in water (100 ml, 30 ml and 30 ml respectively) and dialysed. Fractions I and III were freeze dried. Fraction II was brought to pH 5.0 by the addition of 0.1 times its volume of NH_4Ac buffer (0.2 *M*, pH 5.0) and refractionated with acetone. Precipitates were collected at 40 %, 60 % and 80 % acetone (v/v) (Fractions IIa, IIb and IIc respectively), dissolved in water (30 ml), dialysed and freeze dried. From Table II, which shows the recovery and purification during a typical preparation, it is seen that acetone fractionation can lead to appreciable further purification of the enzyme, Fraction IIb having the highest potency and representing a purification factor of 23 and a recovery, based on a saline-acetone extract, of 49 %.

Adsorption on DEAE cellulose

Further purification of this material was effected in the following way (*cf.* ref. 7). A sample of Fraction IIb (400 mg; 300 units/mg) was dissolved in 200 ml

CO_2-free water. DEAE cellulose (Brown Type-20, 2 g) was added and the suspension stirred gently for 15 min at 0° and then centrifuged. The precipitate was washed with 50 ml CO_2-free water. The combined supernatant and washing was freeze dried and found inactive. The precipitate was resuspended in 200 ml CO_2-saturated water and stirred gently for 15 min at 0° in an atmosphere of CO_2. The suspension was centrifuged and the precipitate washed with 100 ml and then with 50 ml CO_2-saturated water. The combined supernatant and washings was freeze dried.

This material (Fraction C1; 168 mg) assayed at 550 units/mg. Fraction C1 was dissolved in water (5 ml) and applied to a column of DEAE cellulose (1.5 cm in diameter, 12 cm high) equilibrated with CO_2-free water. The column was eluted first with CO_2-free water. After 25 × 2 ml fractions had been collected the eluent was changed to NH_4Ac buffer (0.02 *M*, pH 5.0). A main protein peak appeared between tubes 50 and 60. These fractions were pooled, dialysed and freeze dried. This material (Fraction C2; 80 mg) assayed at 880 units/mg.

The overall purification and recovery data are shown in Table II.

pH-Activity curve

Fig. 2 shows that the purified enzyme has only one peak of activity, *i.e.* between pH 3.5 and pH 4.0, when tested with both bovine haemoglobin and bovine serum albumin. Solutions containing four times this concentration of the purified enzyme showed no activity in the alkaline range.

Activators

The effect of incubation with Ba^{++}, Ca^{++}, Co^{++}, Fe^{++}, Mg^{++}, Mn^{++}, Zn^{++}, ascorbic acid, cysteine and KCN on the activity of adrenal acid proteinase was tested. The enzyme was pre-incubated for 2 h at pH 3.5 in the presence of 0.01 *M* concentra-

TABLE II

RECOVERY AND PURIFICATION OF ACID PROTEINASE FROM A SALINE–ACETONE EXTRACT OF ADRENAL GLANDS

Stage of purification	*Units*/mg protein*	*Recovery (% of units in saline–acetone extract)*	*Purification factor*
Saline extract	9**	63***	
After Step 1 (saline–acetone extract)	13**	100§	1
After Step 2	12**	92	1
After Step 4	51**	84	3.9
After Step 5	70**	84	5.4
Acetone fractionation			
Fraction II	—	68	—
Fraction IIb	300	49	23
Adsorption on DEAE cellulose			
Fraction C1	550	39	42
Fraction C2	880	30	68

* For definition see MATERIALS AND METHODS.

** Calculated from $\frac{\text{units/mg N.}}{6.25}$

*** Total units in saline extract, 334,000.

§ Total units in saline-acetone extract, 533,000.

tions of the various substances and the solutions then assayed against haemoglobin in the usual way (pH 3.5). None of these substances had any effect on the activity of the enzyme.

The enzyme was also pre-incubated for 15 min at pH 3.5 with and without diisopropylfluorophosphate (DFP; 10^{-4} M) and then assayed against haemoglobin. The DFP was without effect.

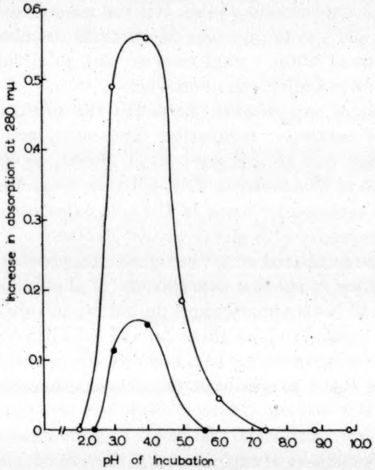

Fig. 2. Variation with pH in proteinase activity of purified adrenal acid proteinase (Fraction C2; Table II). Incubation: 30 min, 37°. ○—○, substrate–bovine haemoglobin; ●—●, substrate–bovine serum albumin.

Action on protein substrates

Solutions of purified enzyme containing 20 units/ml were tested against various protein substrates. The substrates (1 g) were dissolved in water, adjusted to pH 1–2 with 2 M HCl and allowed to stand for 30 min. The solutions were then adjusted to pH 3.5 with 2 M NaOH and made up to 50 ml with water. The activity of the enzyme against these substrates was measured in the usual way. The enzyme was approximately 0.3 as active against bovine serum albumin as against bovine haemoglobin and only about 0.05 as active against human serum albumin. The enzyme apparently had no action on egg albumin, human γ-globulin or porcine thyroglobulin. It should be noted that the method of assay measures only the increase in the number of trichloroacetic acid-soluble fragments produced during hydrolysis which contain those amino acids absorbing at 280 mμ; fragments not absorbing in this region would not have been detected.

Electrophoretic behaviour

Electrophoresis of the purified enzyme on starch gel at pH 8.48 (0.023 M borate; 6 V/cm; 4.5 h) revealed the presence of a major band and two minor slower-moving components.

Peptidase activity on various substrates

The action of a solution of purified proteinase on a number of synthetic peptides and peptide derivatives was tested and compared with the action of a dialysed saline-acetone extract (Step 1, Table II). Using bovine haemoglobin as substrate, the solution of the purified enzyme assayed at 360 units/ml and the crude extract at 90 units/ml.

In the first set of experiments (Table III) the substrates were dissolved in Na citrate buffer (0.1 *M*, pH 5.1) to give 0.04 *M* solutions. Incubation tubes contained: (a) 30 μl substrate; 60 μl buffer; 30 μl enzyme. (b) 30 μl substrate; 30 μl buffer; 30 μl cysteine (0.016 *M* in buffer); 30 μl enzyme.

In the second set of experiments (Table IV) the substrates were dissolved in water to give 0.04 *M* solutions. Incubation tubes contained: (a) 30 μl substrate; 30 μl Na citrate buffer (0.1 *M*, pH 5.1); 30 μl $MnSO_4$ (0.004 *M*); 30 μl enzyme. (b) 30 μl substrate; 30 μl Tris buffer (0.1 *M*, pH 7.8); 30 μl $MnSO_4$ (0.004 *M*); 30 μl enzyme. ($MnSO_4$ was replaced by 0.004 *M* $CoCl_2$ in experiments with glycyl–glycine and by buffer in experiments with glycyl–glycyl–glycine.)

The mixtures were incubated at 37° in tightly stoppered tubes for 16 h.

Following incubation in the first experiments 30 μl of buffer was added to tubes (a) and 30 μl of 0.02 *M* N-ethylmaleimide[8] (in buffer) to tubes (b). 30 μl of 0.06 *M* N-ethylmaleimide was added to both tubes (a) and (b) when the substrate contained cysteine. No additions were made in the second set of experiments.

Samples (5 μl) of the mixtures were then chromatographed together with appropriate controls.

As can be seen from Table III the saline–acetone extract hydrolysed all the substrates tested in the first set of experiments in the presence or absence of cysteine. In the second set of experiments (Table IV) it hydrolysed all the substrates at alkaline pH and also hydrolysed L-leucine amide, glycyl–L-proline, glycyl–glycyl–glycine, L-leucyl-L-phenylalanine and L–leucyl–L-tyrosine at pH 5.1. The purified enzyme had no action under any of the conditions on any of the substrates tested.

TABLE III

PEPTIDASE ACTIVITY OF ADRENAL EXTRACTS AND PURIFIED ADRENAL ACID PROTEINASE

Incubation: 16 h, 37°, pH 5.1. (1) (2) (3) Cathepsin A, B, C substrates respectively[9]. (4) Carboxypeptidase substrate[10]. (5) Thyroid "cysteinyltyrosinase" substrates[11]. ++++, 100% hydrolysis; ++, approximately 50% hydrolysis; —, no hydrolysis.

Peptide	*Saline-acetone extract*		*Purified proteinase 880 units/mg*	
	No activator	*0.004 M cysteine*	*No activator*	*0.004 M cysteine*
Carbobenzoxy-L-glutamyl-L-phenylalanine (1)	++++	++++	—	—
Carbobenzoxy-L-glutamyl-L-tyrosine (1)	++	++	—	—
L-cysteinyl-L-tyrosine (5)	++	++	—	—
Carbobenzoxy-glycyl-L-phenylalanine (4)	++	++	—	—
Glycyl-L-phenylalanine amide* (3)	++	++	—	—
L-cysteinyl-L-tyrosine amide** (5)	++	++	—	—
Benzoyl-L-arginine amide (2)	++	++	—	—

* Gave glycyl-L-phenylalanine.
** Gave cysteine and tyrosine amide.

TABLE IV

PEPTIDASE ACTIVITY OF ADRENAL EXTRACTS AND PURIFIED ADRENAL ACID PROTEINASE

Incubation: 16 h, 37°, 0.001 M $MnSO_4$. (1), leucine aminopeptidase substrates[12]; (2) tripeptidase substrate[13]. ++++, 100% hydrolysis; ++, approximately 50% hydrolysis; +, slight hydrolysis; —, no hydrolysis.

Peptide	Saline-acetone extract		Purified proteinase 880 units/mg	
	pH 5.1 (citrate)	*pH 7.8 (Tris)*	*pH 5.1 (citrate)*	*pH 7.8 (Tris)*
L-leucine amide (1)	++	++++	—	—
Glycyl-L-proline	++	++++	—	—
Glycyl-L-phenylalanine	—	++++	—	—
Glycyl-L-tyrosine	—	++++	—	—
Glycyl-L-leucine	—	++++	—	—
Glycyl-glycine*	—	++	—	—
Glycyl-glycyl-glycine** (2)	++	++++	—	—
L-alanyl-L-alanine	—	++++	—	—
L-alanyl-L-leucine (1)	+	++++	—	—
L-leucyl-L-phenylalanine (1)	++	++++	—	—
L-leucyl-L-tyrosine (1)	++	++++	—	—

* $CoCl_2$ replaced $MnSO_4$.
** Buffer replaced $MnSO_4$.

DISCUSSION

A comparison of the proteinase activities at pH 3.5 (haemoglobin substrate) of extracts of endocrine glands, liver and muscle showed that, with the exception of muscle, all the extracts had considerable activity. The acid proteinase activities/mg N of thyroid extracts were comparatively low, although presumably proteolytic enzymes are involved in the release of thyroid hormone from thyroglobulin, its stored form in the gland. In contrast, the values for ovary and for adrenal extracts at this pH were high and of the same order as those for liver. Since the hormones of both adrenal and ovary glands are not protein in nature, nor stored in protein form, it would appear that acid proteinases play a general rather than a specific role in cell metabolism.

The pH-activity curves (haemoglobin substrate) of extracts of thyroid and of adrenal glands resembled those of extracts of anterior pituitary glands[1] in that they each showed two pH optima—at pH 3.5 to pH 4.0 and at pH 8.5 to pH 9.5. Also, the peptidase activities of beef adrenal extracts (see Tables III and IV) were similar to those reported for pituitary extracts[1].

The purified adrenal acid proteinase, like both purified thyroid acid proteinase[2–4,14] and pituitary Proteinase I (see ref. 1), was not activated by metal ions or reducing agents nor did it hydrolyse a wide range of synthetic peptide substrates including those for Cathepsins A, B and C (see Table III). On the other hand, pituitary Proteinase I has been found to hydrolyse human γ-globulin[1], whereas the adrenal acid proteinase had apparently no action on this substrate. Additional studies of the action of the three acid proteinases on purified protein substrates may well reveal other specificity characteristics.

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PREPARATION OF BACTERIAL ENZYMES BY CONTROLLED LYSIS

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SUMMARY

1. Lysis of *Vibrio cholerae* and *Escherichia coli* under controlled conditions by Versene and lysozyme has been adapted to separate the cells into three major fractions.

2. Succinic oxidase, cytochrome oxidase and DPNH-cytochrome *c* reductase are located in the protoplast membrane or sediment.

3. TPN linked *iso*-citrate, G6P dehydrogenases, DPN-linked malic dehydrogenase, aldolase, aspartic acid deaminase, adenosine deaminase, histidase and *iso*-citritase activities are present in the lysates of the "protoplasts" obtained by this method.

INTRODUCTION

Extracts of bacteria for enzymic and antigenic analysis are usually prepared by rupturing the cells either by freezing and thawing, grinding with abrasives, extrusion under mechanical pressure or by exposure to high frequency sound waves.[1,2]. Intermixing of the constituents of the different anatomical structures of the cell such as the cell wall, the protoplast membrane and the cytoplasm is unavoidable in the use of these techniques. Preparations obtained by these procedures are, therefore, only of limited use in studies pertaining to localization or classification of enzymes and antigens on a morphological basis. Further, these drastic methods of cell disruption cause extensive damage to coupled enzyme systems[3]. A certain degree of denaturation of polymer constituents, resulting in the loss of their biological activity, is inevitable under these conditions.

The present study was undertaken with the object of devising a method free from the above limitations for the preparation of enzymically active cell-free extracts of bacteria. The results obtained with *Vibrio cholerae* and *Escherichia coli* indicate that controlled lysis by the combined action of metal complexing agents and lysozyme could be used to yield preparations suitable for localization and fractionation of enzymes.

MATERIALS AND METHODS

Organisms

Vibrio cholerae – Inaba-6-S^S and Inaba-6-S^R and *Escherichia coli* – M 133-3 were employed in this study.

Media

Nutrient agar (pH 7 for *E. coli* and pH 8 for *V. cholerae*) were used for the maintenance and propagation of the cultures.

Chemicals and reagents

Lysozyme (egg white, crystalline), diphospho- and triphospho-pyridine nucleotides (DPN and TPN) and the barium salts of glucose-6-phosphate (G6P) and hexose diphosphate (HDP) were procured from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U. S. A.; Versene from Eastman Organic Chemicals, Rochester, New York, U. S. A. and tris(hydroxymethyl)aminomethane (Light Chemical Company, Colnbrook, Bucks, England) was a gift from Dr. E. M. CROOK of the Biochemistry Department, University College, London. Dimethyl-d-*iso*-citrate lactone prepared originally by Dr. G. W. PUCHER from *Bryophyllum* leaves was kindly supplied by Dr. P. S. KRISHNAN, Professor of Biochemistry, Lucknow University, Lucknow, India. The ester was converted into the potassium salt of *iso*-citric acid by heating with a slight excess of 1 *N* potassium hydroxide in a water bath for 30 min and adjusting the pH of the resulting solution to 7 with 1 *N* hydrochloric acid. The barium salts of G6P and HDP were converted into their respective potassium salts by removal of barium as sulphate by the addition of the requisite amount of potassium sulphate.

Centrifugations were carried out in the Spinco ultracentrifuge Preparative Model L and optical density measurements were made in the Beckman spectrophotometer Model DU.

Enzyme assays were made by standard methods as indicated below:

Adenosine deaminase according to KORNBERG AND PRICER[4].

Aldolase according to SIBLEY AND LEHNINGER[5].

Aspartase according to IYER *et al*[6] or by following the increase in optical density at 300 mμ due to the formation of fumarate. This system was found to be valid for preparations free from fumarase.

DPNH-cytochrome reductase photometrically, using triphenyl tetrazolium chloride (TTC) as hydrogen acceptor[7]. The formazan was extracted and estimated according to SRIKANTAN AND KRISHNA MURTI[8].

Cytochrome oxidase manometrically using ascorbate as the reducing agent.

Histidase according to MEHLER AND TABOR[9].

G6P, *iso*-citrate and malic dehydrogenase spectrophotometrically at 25° following the increase in optical density at 340 mμ of reduced TPN and DPN. *Iso*-citritase according to OLSON[10]. Succinic oxidase manometrically. Protein concentration in extracts was estimated by the spectrophotometric method of WARBURG AND CHRISTIAN[11].

Preparation of cells

Harvesting and preparation of the cells for lysis was carried out by a method routinely employed in this laboratory[12].

Controlled lysis of cells

Freshly harvested and washed cells were suspended in 0.85 % sodium chloride to an opacity value corresponding to 10–20 % light transmission (Red filter 650 mμ) in the Lumetron colorimeter. The saline suspension of cells was added to a mixture of Tris–hydrochloric acid buffer (pH 8), versene, lysozyme and sucrose. In a typical experiment the constituents in a total volume of 600 ml were: 200 mg versene, 40 mg lysozyme, 100 ml of 0.1 *M* Tris–HCl buffer, 160 ml of 2 *M* sucrose and 100 ml of cell suspension. The flask containing the above reaction mixture was incubated at 38° with gentle shaking for 2–3 h. Within this period the majority of cells are usually found to assume spherical shapes when viewed through a high power microscope. The suspension was left overnight at 5–8°. The subsequent operations are summarized in Fig. 1. The term "protoplasts" is used here in the limited sense as applicable to Gram-negative organisms[13].

Extensive lysis occurred on suspending the "protoplasts" in chilled water yielding a very viscous suspension. High speed centrifugation of this suspension was facilitated by exposing the lysate for 2–5 min to sound waves (25 kc/sec with an output amperage of 2.5 generated in a Mullard Magnetostrictor Oscillator).

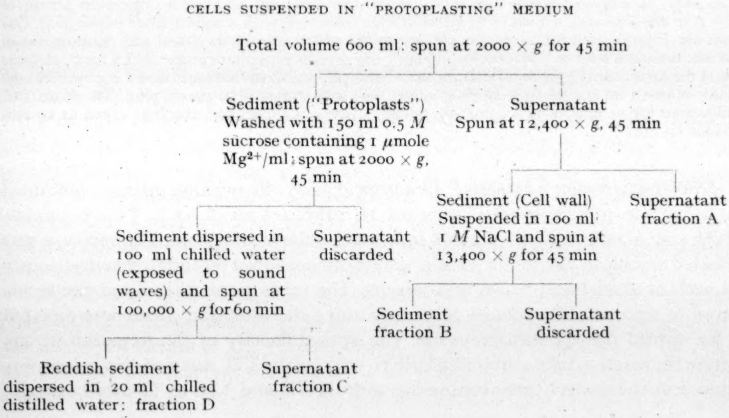
CELLS SUSPENDED IN "PROTOPLASTING" MEDIUM
Total volume 600 ml: spun at 2000 × g for 45 min
Sediment ("Protoplasts") Washed with 150 ml 0.5 M sucrose containing 1 µmole Mg2+/ml; spun at 2000 × g, 45 min
Supernatant Spun at 12,400 × g, 45 min
Sediment dispersed in 100 ml chilled water (exposed to sound waves) and spun at 100,000 × g for 60 min
Supernatant discarded
Sediment (Cell wall) Suspended in 100 ml 1 M NaCl and spun at 13,400 × g for 45 min
Supernatant fraction A
Sediment fraction B
Supernatant discarded
Reddish sediment dispersed in 20 ml chilled distilled water: fraction D
Supernatant fraction C

Fig. 1. A scheme for the fractionation of bacterial cells by lysis.

RESULTS

The four fractions (A, B, C and D) resulting from the controlled lysis of the cells were assayed for enzyme activity and their optical densities were read over the range 230 to 300 mμ after suitable dilution. Fraction A showed no absorption of u.v. light, fraction B exhibited a high absorption at 230 mμ and a sharp decline in absorption thereafter. Absorption curves of C and D were humped in the range 250–265 mμ characteristic of nucleoproteins. Fractions A and B were devoid of all the enzyme activities tested. Fraction D showed considerable activity with respect to cytochrome oxidase, succinic oxidase and DPNH cytochrome reductase but none of the other enzymes. Fraction C while not possessing the above activities was rich, however, in adenosine deaminase, aspartase, aldolase, histidase and the pyridine nucleotide linked dehydrogenases.

Enzyme activities of fraction D

Succinic oxidase and cytochrome oxidases: The results of the assay of these two enzymes are given in Table I. These observations support the earlier findings of SAGAR *et al.*[14] that the succinic oxidase activity of *V. cholerae* is localized mainly in the particulate fraction.

TABLE I

SUCCINOXIDASE AND CYTOCHROME OXIDASE ACTIVITIES OF FRACTION D

	Enzyme protein (mg/flask)	*µl oxygen uptake in 10 min*	
		Succinic oxidase	*Cytochrome oxidase*
Inaba-6-S^S	12.8	14.8	26.8
Escherichia coli	8.4	17.2	64.0

Flask contents: succinic oxidase: 100 µmoles phosphate buffer (pH 8 for *V. cholerae* and pH 7.2 for *E. coli*); 10 µmoles Versene (pH 7.5), cytochrome *c* 100 µg; 50 µmoles potassium succinate (pH 7.5) in the side arm. 0.2 ml 10% KOH in the centre well with a square filter paper plug. Gas phase: air. Equilibrated for 15 min at 38°, succinate added, stopcocks closed and readings taken at 10 min intervals for 1 h. Cytochrome oxidase: 100 µmoles phosphate buffer (pH 8 for *V. cholerae* and pH 7.2 for *E. coli*); 33 µmoles sodium ascorbate (pH 7.5) in the side arm and 1 mg cytochrome *c*; total volume 3 ml. 0.2 ml 10% KOH in centre well with square filter paper, plug. Gas phase: air. Equilibrated for 15 min at 38°, ascorbate added, stopcocks closed and readings taken at 10 min intervals for 1 h.

DPNH-cytochrome c *reductase*: In a typical assay the reaction mixture contained 1 ml of sodium pyrophosphate buffer 0.1 *M*, pH 8, 0.5 ml of 0.5% TTC, 0.3 µmole DPNH and 8.0 mg enzyme protein in a total volume of 3.0 ml. The mixture was incubated aerobically at 38° for 30 min and reaction stopped by adding simultaneously 5 ml each of glacial acetic acid and toluene. The tubes were shaken and the layers allowed to separate. The toluene layer containing the formazan colour was pipetted out for optical density measurements. The optical density of the formazan at 495 mµ from the reaction tube containing DPNH, enzyme and TTC was 0.08. No colour was produced in the control tubes containing only DPNH and TTC or TTC and enzyme.

Enzyme activities of fraction C

Adenosine deaminase: Reaction mixtures consisting of 100 µmoles of Tris–HCl buffer (pH 8), 10 µmoles Mg^{++} as the chloride, 10 µmoles of adenosine and 0.2 ml of fraction C (750 µg protein) in a total volume of 2 ml were incubated at 38° for 30 min after which 2 ml 10% trichloroacetic acid were added. To the control tube, 10 µmoles of adenosine were added immediately after the addition of the trichloroacetic acid. The tubes were spun at 1800 *g* for 30 min and the optical densities of the supernatants read at 255 mµ after diluting 100 fold with distilled water. The O.D. values for the control and experimental tubes were 0.375 and 0.015 respectively indicating that over 95% of the adenosine had been deaminated by the enzyme in 30 min.

Aspartic acid deaminase: Reaction mixtures consisting of 100 µmoles of Tris–HCl buffer (pH 8), 10 µmoles of Mg^{2+} as the chloride, 20 µmoles of DL-aspartic acid (pH 7.5), 0.2 ml fraction C (750 µg protein) in a total volume of 2 ml were incubated at 38° for 60 min and ammonia determined in the filtrate obtained by deproteinization with an equal volume of 10 % trichloroacetic acid. 58 μg of ammonia-N were liberated in the experimental tube accounting for about 40 % deamination of the substrate.

HDP-aldolase: Results of the aldolase assay of fraction C of *Vibrio cholerae* and *Escherichia coli* are given in Table II from which it can be seen that apart from possessing considerable HDP-alodolase activity, the extracts are apparently activated by cysteine. This is in conformity with observations on *V. cholerae*, *Pasteurella pestis*, and *Salmonella typhosa* reported earlier from these laboratories[15–17].

TABLE II

HDP-ALDOLASE ACTIVITY OF FRACTION C OF *Vibrio cholerae* AND *Escherichia coli*

	Inaba-6-S^S	*Inaba-6-S^R*	*A. coli*
Without any activator	183	186	162
With 10 μmoles cystein-HCl	705	710	786

Results are expressed as Klett units (540 mμ) of the coloured dinitrophenyl hydrazones of the trioses formed in 1 ml filtrate. Reaction mixture: 100 μmoles Tris–HCl buffer pH 8.6; 140 μmoles hydrazine sulphate (pH 8.6); 6.5 μmoles HDP and 750, 720 and 1200 μg respectively of the enzyme protein in In-6-S^S, In-6-S^R and *E. coli* in a total volume 2.5 ml.

Histidase: Silica cuvettes (1 cm optical path) contained in a total volume of 3 ml, 100 μmoles phosphate buffer (pH 8), 5 μmoles reduced glutathione, 10 μmoles DL-histidine and 660 μg enzyme protein (Inaba-6-S^S). Increase in optical density at 277 mμ was followed against a reference cell containing all the reactants excepting histidine. The optical density reached a maximum value of 1.1 within this period. Specific activity of the extract was found to be 45 units/mg protein (1 unit of enzyme is the amount that causes an increase in O.D. at 277 mμ of 0.001/min[9]).

Iso-citritase: 3 ml of reaction mixture in silica cuvettes contained, in μmoles, cysteine-HCl 6; semicarbazide-HCl, 60; Mg^{2+} as the chloride 16; phosphate buffer (pH 8) 200; *iso*-citrate 10 and enzyme protein 280 μg. The increase in O.D. at 252 mμ was followed. The reaction started after a lag period of 6 min and the O.D. reached a value of 0.35 in 85 min giving a specific activity of 9 units (1 unit is the amount

TABLE III

DEHYDROGENASE ACTIVITIES OF FRACTION C

Organism	*Substrate (10 μmoles)*	*Enzyme protein in test system (μg)*	*DPN (μmoles)*	*TPN (μmoles)*	*Specific activity (units)*
In-6-S^S	Malate	750	0.3	—	130
Escherichia coli	Malate	1200	0.15	—	75
In-6-S^S	G6P	750	—	0.25	86
In-6-S^R	G6P	800	—	0.125	28
In-6-S^S	*Iso*-citrate	375	—	0.25	240
In-6-S^R	*Iso*-citrate	400	—	0.125	140

1 unit of enzyme is the amount that causes the increase in O.D. at 340 mμ of 0.001 in 1 min. Reaction mixture: 150 μmoles Tris–HCl buffer (pH 8 for *V. cholerae*; 7 for *E. coli*) substrate and enzyme as indicated in table; 10 μmoles of Mg^{2+} as the chloride in a total volume of 3 ml. Reaction started by the addition of the co-enzyme. The test system for malic dehydrogenase included 50 μmoles cyanide (pH 8) in addition to the above constituents.

of enzyme that causes an increase in O.D. at 252 mμ of 0.01 in the first 5 min after reaction started). Indirect evidence for the presence of this enzyme and hence the glyoxalate shunt in *V. cholerae* has been reported elsewhere on the basis of the isolation of glyoxalate as a dinitrophenylhydrazone identified chromatographically[18].

Dehydrogenases: Results of the assay of *iso*-citrate-TPN, G6P-TPN and malic acid-DPN dehydrogenases are summarized in Table III.

DISCUSSION

The method of lysis under controlled conditions as devised in this study makes it possible to effect, by a comparatively gentle technique, the separation of Gram-negative bacteria into three major components: *viz.*, a major part of the cell wall devoid of any enzyme activity, the "protoplast" membrane together with other 100,000 *g* sedimentable particles and a supernatant. Three of the pyridine nucleotide linked dehydrogenases, aspartase; adenosine deaminase, histidase and *iso*-citritase activities of *Vibrio cholerae* are present in the supernatant. Cytochrome oxidase, succinic oxidase and DPNH-cytochrome *c* reductase are, on the other hand, associated with the membrane fraction. Although only a few enzymes have been investigated in the present study, the distribution of their activities in the membrane fraction and the supernatant resembles broadly the distribution pattern reported for mitochondrial and supernatant fractions of rodent liver homogenates[19].

The membrane fraction obtained by controlled lysis is likely to retain part of the cell wall and also to be contaminated with cytoplasmic inclusions sedimentable at 100,000 *g*. By differential centrifugation this fraction can be resolved further into components of decreasing particle size. Similarly by the use of conventional techniques such as ammonium sulphate fractionation and calcium phosphate gel adsorption and elution or ion-exchange chromatography, the supernatant can be separated into individual enzymes.

Exploratory immunochemical analysis by gel diffusion technique (S.B. MISRA and D. L. SHRIVASTAVA, unpublished observations) has revealed that the cell wall fraction, the lysate and the lysate sediment give rise to precipitin lines with *Vibrio cholerae* anti-sera. While the cell wall fraction gives only one line, fractions C and D produce multiple lines. Also fraction D is found to give rise to a line corresponding to the one shown by the cell wall fraction. This is in conformity with the findings of other workers that the "protoplast" membrane of Gram-negative organisms retains part of the cell wall[20].

Although lysozyme was used by PENROSE AND QUASTEL[21] as early as 1930 to compare activities of certain enzymes of intact cells and extracts of *Micrococcus lysodeikticus*, techniques based on controlled lysis have not been employed hitherto for enzyme localization. Some attempts have, however, been made recently to locate in the lysates of *Escherichia coli* protoplasts[22], lysates of *Bacillus megatherium* protoplasts[23] and the 20,000 *g* sedimentable membrane fraction of *Alcaligenes faecalis*[24], the cellular activity that normally mediates the incorporation of labelled amino acids into structural proteins. Likewise over 90 % of the oxidative activity of *Staphylococcus aureus* is associated with the plasma membrane fraction[25]. STANIER[26], on the basis of WEIBULL's findings[27] suggests that the bacterial cytochrome system, along with other enzymes, may be a built in part of the cell membrane. The observations tend to show that corresponding to the well-known differentiation of tissues with respect to function in higher organisms, discrete compartments of the bacterial cell are presumably equipped with enzymic mechanism to mediate specialized functions such as electron transport, oxidative phosphorylation or protein synthesis[28]. The importance of obtaining further insight into the structural organization of the cell from the point of view of drug action has been emphasised[29]. It is in this context that the practical value of the technique of controlled lysis as suggested in the present study assumes added significance.

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PURIFICATION AND PROPERTIES OF GLUCOSE DEHYDROGENASE AND CYTOCHROME *b* FROM *BACTERIUM ANITRATUM**

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SUMMARY

A soluble glucose dehydrogenase with a turnover number of 28,000 moles of glucose/min per 10^5 g of protein has been purified from *Bacterium anitratum*. The enzyme does not posess a flavin or heme prosthetic group. It catalyzes the reduction of 2,6-dichlorophenolindophenol and phenazine methosulphate well, but ferricyanide and methylene blue only very slowly. Reduction of DPN, TPN, FMN, FAD, cytochrome *c*, tetrazolium, or oxygen could not be detected.

The dehydrogenase was accompanied during all purification steps except the last by a *b*-type cytochrome with absorption maxima at 419 and 530 mμ in the oxidized state, 428, 532, and 562 mμ in the reduced. It was purified to the stage of $2.9 \cdot 10^5$ g protein per mole of protoporphyrin. In the presence of the dehydrogenase and possible unidentified linking factors, the hemoprotein was reduced by glucose.

Evidence is presented and the possibility discussed that the two proteins are soluble precursors or degradation products of the particulate glucose oxidizing system from the same organism.

INTRODUCTION

Oxidation of glucose to gluconic acid is carried out by many bacteria, notably by *Acetobacter*, *Pseudomonas*, *Aerobacter*, and *Azotobacter* species[2–6]. In these organisms glucose oxidation has been found linked to structural elements, in some instances shown to carry cytochromes reducible by glucose[2,3]. No evidence has here been found for the intervention of DPN or TPN in the primary dehydrogenation. A few instances are, however, known where bacteria or their spores contain a soluble glucose dehydrogenase resembling the mammalian liver enzyme in being DPN-linked[7,8]. The present communication describes a soluble glucose dehydrogenase which is independent of externally added DPN or TPN for its action. It is purified from a bacterial homogenate, where a large part of the dehydrogenase activity is associated with particles in the manner usually found.

Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; DIP, 2,6-dichlorophenolindophenol.

* A brief note on the early phase of this work has appeared elsewhere[7].

METHODS

Microorganism

The strain of *Bacterium anitratum** used was isolated in this laboratory and kindly identified by Dr. H. LAUTROP, Statens Seruminstitut, Copenhagen. It was grown under vigorous aeration and agitation in a 25 l container at 37°, either in batch or continuous culture. The medium contained 4.7 g Difco Casamino Acids, 4.7 g glucose, 5.5 g Na_2HPO_4, 1.7 g K_2SO_4, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.8 mg $FeSO_4 \cdot 7H_2O$, and distilled water to 1 l. During the growth pH was kept between 6.9 and 7.4 by addition of ammonium hydroxide. The cells were harvested at a density of 1.0–1.5 g dry wt./l in a super centrifuge, and washed, when required, by resuspension in several liters of distilled water, followed by a second centrifugation. The cell paste was then dried from the frozen state and stored at —20°. It was usually found, for reasons yet to be investigated, that the cells grown in batch process gave a higher yield of soluble enzyme per gram of cells than the cells from continuous culture.

Preparation of cell-free extract

The cells were disintegrated by grinding with Ballotini glass beads, No. 12, in a MSE homogenizer cooled with ice-water. 5 g of cells and 20–25 ml of water were added to 40 ml of wet glass beads, and the homogenizer run at close to full speed for 2 × 8 min. As the cells were broken, the mass turned from a thick to a thin paste. This paste was then diluted with 20–30 ml water, and centrifuged for 5 min at 24,000 × *g* in the cold. The sediment was suspended in 30 ml 0.1 *M* phosphate buffer, pH 6.0, and recentrifuged. The two supernatants were combined and centrifuged for 90 min at 24,000 × *g*. A well packed layer of cells and heavy fragments, a suspended layer of smaller fragments near the bottom, and a clear supernatant, showing Tyndall effect, were obtained. The clear supernatant represents the starting material for the enzyme studies. 40–50 ml with 600–700 mg of protein was obtained from 5 g of cells.

Analytical methods

Protein was measured with the biuret method[12], with the addition of 20 μl 10% desoxycholate per 3 ml of reaction mixture when lipoprotein complexes were present. The mixture then had to be centrifuged before reading of the O.D. In purer preparations, the protein concentration was calculated from the absorptions at 260–280 mμ.

Glucose was determined by the anthrone method[13], and gluconolactone determined as hydroxamate with authentic D(+)-gluconolactone as standard, according to HESTRIN[14].

Aerobic oxidation was followed in the Warburg apparatus at 30° with air as the gas phase, in a medium of 0.05 *M* phosphate, pH 6.0. Dehydrogenase activity was measured spectrophotometrically in a Beckman DU at 25° by reduction of DIP in 3 ml total volume in 1 cm cuvettes with an initial O.D. at 600 mμ of 0.6. The buffer was as above, and glucose was added to an initial concentration of 0.02 M. The reaction was started by the stirring in of the enzyme preparation. Enzyme activities were calculated based on the time needed for an initial O.D.-decrease of 5–10 %. 1 unit of enzyme is defined as the amount giving an O.D.-reduction of 1/min under these standard conditions, and specific activity is equal to units/mg of protein. Reaction rates in terms of μmoles DIP reduced were computed based on an extinction coefficient of $21.0 \cdot 10^6$ cm^2 × mole^{-1} at pH 7.0 (see ref.[15]), which at pH 6.0 corresponds to a value of $14.9 \cdot 10^6$ (experimentally determined).

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* *Bacterium anitratum* is a commonly found apathogenic organism whose position in the taxonomic system has not yet been finally agreed upon[9,10]. It has many similarities to species within the family *Achromobacteraceae*, particularly *Alcaligenes viscolactis* and *Achromobacter butyri*. BRISOU AND PREVOT[11] have suggested a new arrangement and definition of *Achromobacteraceae*, in which among others these two species and *Bacterium anitratum* fall in a common new genus denoted *Acinetobacter*. The present genus *Alcaligenes* is proposed eliminated.

Other electron acceptors were tested under the same substrate, volume, buffer, and pH conditions. Cytochrome *c* and ferricyanide reductions were measured at 550 and 418 mμ respectively, using extinction coefficients of $19.1 \cdot 10^6$ and $1.02 \cdot 10^6$ respectively. Reduction of methylene blue, triphenyl tetrazolium chloride, FAD and FMN were studied in Thunberg tubes *in vacuo*.

The presence of flavin was investigated in the following manner: 125 μg of highly purified enzyme in 250 μl phosphate buffer, pH 6.0, was denatured by brief heating to 100° and treated with 50 μg Difco Trypsin, added in 20 μl 0.5 M Na_2HPO_4, for 1 h at 37°. The sample was again heat-denaturated and precipitated with 40 μl 50 % trichloroacetic acid. The absorption spectra were recorded at the various stages in this procedure, following clarification by centrifugation. The final supernatant was adjusted to pH 2.7 with ammonia, and the fluorescence spectrum recorded in the micro-cuvette of a Farrand spectrophotofluorimeter, the excitation beam having a wavelength of 365 mμ.

Pyridinohemochromogens were prepared by making the hemoprotein solution 0.1 N with respect to NaOH, and adding 1/3 volume of pyridine. They were reduced with $Na_2S_2O_4$. As the extinction coefficient for the α-band of protohemin hemochromogen[16] $34.8 \cdot 10^6$ was used.

Absorption spectra were recorded in volumes of 0.20–0.25 ml in semi-micro cuvettes, the Beckman DU being equipped with an appropriate diaphragm.

MATERIALS

Ballotini glass beads were obtained from H. Mickle, Gomshall (Surrey); D-gluconolactone from Hoffmann-La Roche and Co; N,N-diethylaminoethylcellulose (DEAE-cellulose) from Eastman Kodak Company; carboxymethylcellulose (CM-cellulose) from Serva Entwicklungslabor; triphenyltetrazolium chloride, crystalline bovine serum albumin, and FAD (80 % pure) from Nutritional Biochemicals Corporation; horse heart cytochrome *c*, reduced DPN (90 % pure), FMN, TPN (95 % pure), *p*-chloromercuribenzoate, and phenazine methosulphate from Sigma Chemical Company; DPN from Pabst Laboratories; trypsin 1:250 from Difco Laboratories; atebrin from Bayer, Leverkusen; methylene blue from G. T. Gurr, London; 2,6-dichlorophenolindophenol from Hopkin and Williams Ltd.

EXPERIMENTAL AND RESULTS

Purification

The purification was initiated by precipitation with protamin sulphate, which was added in quantities sufficient to reduce the 260 mμ O.D. of the extract from 180 to around 20. This usually was 300 mg for a 5 g cell batch. Inactive protein and a portion of the enzyme activity were also precipitated, giving a supernatant of specific activity close to that of the extract. This first step was necessary for a successful subsequent ammonium sulphate fractionation. This was carried out at pH 6, in steps given in Table I. Some purification was achieved in the 61–76 % saturation fraction. After dialysis for 4 h against 0.005 *M* phosphate, pH 7.0, this fraction was applied to an equilibrated DEAE-cellulose column of 1 g DEAE-cellulose/50 mg of protein, and elution started with the dialysis buffer. The dehydrogenase activity was under these circumstances not bound by the column, while much of the inactive protein remained attached. Concentration and further purification was achieved by adsorption on calcium phosphate gel, 2 mg added/mg of protein. Most of the activity was adsorbed, and eluted again with 0.1 *M* phosphate of pH 6, containing 10 % ammonium sulphate. All steps were carried out at a temperature of 0–5°. The protocol for a purification up to this stage, starting with 25 g of cells from a continuous culture, is summarized in the upper part of Table I.

TABLE I

MAIN PURIFICATION

Purification step	*ml*	*mg protein*	*Units*	*Units/mg*
Extract	238	2,900	1,050	0.36
Protamin sulphate supernatant	250	2,300	780	0.35
Ammonium sulphate: 0–45 %	16	240	100	0.41
45–55 %	36	1,140	300	0.29
55–61 %	4.3	160	40	0.24
61–76 %	3.7	115	100	0.91
Supernatant	220	460	0	0
DEAE-cellulose eluate	15	5.5	102	18
Calcium phosphate gel eluate	1.9	2.0	82	41
Ammonium sulphate: 50–55 %	38	530	125	0.24
140,000s240*	31	350	28	0.08
140,000p240	9	93	54	0.58
Semi-sedimented layer	2	60	16	0.27

* Ultra centrifuge supernatant and precipitate fractions, with relative centrifugal force and centrifugation time (min) indicated.

For further purification, a small scale column fractionation on carboxymethyl cellulose was set up. 3–6 mg protein was dialyzed against 0.01 *M* phosphate buffer of pH 6.0 and adsorbed on a column of 100 mg carboxymethyl cellulose that had previously been equilibrated with the same buffer. Elution was carried out with phosphate buffers in the following steps of molarity and pH: 0.01–6.00, 0.02–6.30, 0.03–6.60, 0.04–6.70, 0.05–6.85, 0.06–7.00, and fractions of 0.6 to 0.9 ml collected. The progress of the elution was followed in terms of enzymic activity, protein concentration (280 mμ absorption), and cytochrome content (difference in absorption between 419 and 380 mμ). The dehydrogenase activity was slowly eluted at pH 6.70, and rapidly by the following buffer. In one such fractionation, to be further discussed below, 125 units of enzyme, specific activity 36, were applied to the column. 80 units of specific activity 390 were recovered in the first fractions of the pH 6.85 buffer. The following fractions also contained considerably enriched material. The protein eluted was very dilute, around 0.1 mg/ml. It was concentrated as follows: after 2 h dialysis against 0.01 *M* phosphate of pH 6.0, suitably pooled fractions were led through a bed of 5–10 mg carboxymethyl cellulose, equilibrated as above. 0.3 ml buffer of a pH and molarity somewhat in excess of what was necessary for initial elution was used to reelute the fractions.

Inspection of Table I shows a high loss of enzyme units in the 0–45, and particularly in the 45–55 % ammonium sulphate fraction. Refractionation of the 45–55 fraction did not bring more activity into the 61–76 range. A part of the material precipitating between 50 and 55 % was then subjected to ultra centrifugation at 140,000 × g* in the preparative Spinco centrifuge for 4 h. The lower part of Table I demonstrates that a large part of the activity of this fraction was sedimentable under these conditions. The degree of purification was twofold.

The purification process thus presents us with two different forms of the enzyme. The major part of the glucose dehydrogenase units appears to be linked to small sedimentable particles, and this portion is purified to a small degree only. Another portion of the extract activity is not sedimentable under the above conditions; it behaves like a soluble enzyme in the purification process and is considerably purified. The relative amount obtained of the soluble form varied from one batch of cells to another. Studies on the small particle fraction, purified as above, have been carried out alongside the investigation of the properties of the soluble dehydrogenase, on the hypothesis that the latter might be a degradation product or a precursor of the former, and that they therefore might show some similar traits.

The stability of the enzyme preparations at all stages of purity is fairly good. When kept in the frozen state at ÷ 20°, the decline in activity was on the order of 5–15 % in a month for the soluble enzyme. Occasionally there have been unexplained larger losses at some point of purification or in storage.

Hydrogen acceptors

The original extract oxidizes glucose with oxygen as well as with DIP as hydrogen acceptor, the DIP rate usually being 2–4 fold higher than the rate with oxygen. After fractionation, the ability to react with oxygen is retained in the 45–55 % ammonium sulphate fraction and in the particles isolated from this, whereas it is lost in the 61–76 fraction, and all further purification steps. The high speed precipitate referred to in Table I carried out oxidation of glucose at a rate of 150 μl O_2/mg protein/h, while the equivalent rate with DIP was half this figure.

In addition to DIP and oxygen, TPN, DPN, FAD, FMN, phenazine methosulphate, triphenyltetrazolium chloride, methylene blue, cytochrome *c*, and ferricyanide were tested. The results are given in Table II. The original extract or dialyzed fractions were not stimulated in their DIP reduction by addition of pyridine nucleotides, neither were these reduced when added in the absence of DIP. Cytochrome *c* was not measureably reduced by glucose in the presence of either the dehydrogenase or the particle, unless small amounts of DIP or phenazine methosulphate were present as carrier. Ferricyanide was reduced very slowly by the purified dehydrogenase, and somewhat faster by the particle. Again, DIP was a very effective bridge, and with DIP added, the reduction of ferricyanide proceeded linearly at a rate determined by the DIP concentration, until the ferricyanide remaining was of the same order of magnitude as the DIP added. No reaction with tetrazolium could be detected with either of the two forms. Methylene blue was reduced too slowly to be an effective bridge to oxygen for the soluble enzyme. Phenazine methosulphate, however, coupled the dehydrogenase to oxygen in manometric experiments, at a rate close to the rate obtained with DIP.

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* Centrifugal force at tube bottom.

TABLE II

RELATIVE RATES WITH DIFFERENT HYDROGEN ACCEPTORS

Acceptor	*Concn.** *($M \times 10^5$)*	*Relative rates*	
		Sol. enz.	*Part. enz.*
DIP	4	100	100
Oxygen	(air)	<1	50–200
Phenazine methosulphate	35	100	
Methylene blue	6	2	<2
Ferricyanide	50	0.3	3
FAD, FMN	3	0	0
DPN, TPN	14	0	0
Cytochrome *c*	8	0	0
Triphenyltetrazolium chloride	500	0	0

* These figures represent convenient rather than saturating concentrations. The dependence of the rate on the concentration of the main acceptor DIP is discussed in the accompanying paper[19].

Nature of the product

Resting cells oxidized glucose rapidly to the level of 1 atom oxygen consumed/molecule glucose, and from then on at a reduced rate. In this first period CO_2-evolution was slight. Cell-free extracts and particle preparations oxidized glucose only one step under the present test conditions. This all pointed towards gluconic acid as the probable candidate for the product, particularly since gluconic acid or gluconolactone has been shown to be the product in other particulate glucose oxidation systems[2,4,6].

A manometric experiment was set up with soluble dehydrogenase from the gel eluate level and phenazine methosulphate as link to oxygen, as described in Table III. Two parallel vessels were used, which were removed at different times. The reaction was stopped with trichloroacetic acid, and glucose and gluconolactone determined. It is evident that hydroxamate-forming material was produced in conjunction with

TABLE III

PRODUCT FORMATION

The Warburg vessels contained 150 µmoles phosphate buffer, 37 µg dehydrogenase of specific activity 70, 2.5 mg bovine serum albumin, 20 µmoles glucose (added at zero time), 0.3 mg phenazine methosulphate, and water to 3 ml at a pH of 6.0. The reaction was stopped by addition of 0.5 ml 10 % trichloroacetic acid, and aliquots taken after centrifugation for determination of glucose and gluconolactone. It was necessary to correct for the ability of glucose itself to form some color in the hydroxamic acid test, as well as for the color of decomposed phenazine methosulphate.

Time (min)	*Glucose oxidized (µmoles)*	*Gluconolactone formed (µmoles)*	*Oxygen consumed (µmoles)*
25	11.3	7.1	6.9
60	14.0	8.3	10.0

oxygen uptake and glucose disappearance, and it is assumed that this hydroxamate represents gluconolactone. There is not full correspondence between glucose oxidized and lactone formed. Loss of gluconolactone by spontaneous hydrolysis must, however, be expected to occur during the reaction and handling before the assay. The oxygen uptake is between 1 and 2 atoms per molecule glucose, indicating partial decomposition of the hydrogen peroxide formed during the reoxidation of phenazine.

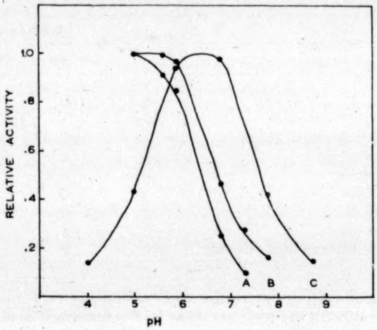

Fig. 1. pH dependence. The following buffers were used: pH 4.1 and 5.0 (acetate); pH 5.9, 6.8 and 7.3 (phosphate); pH 7.8 and 8.7 (tris(hydroxymethyl)aminomethane). Curve A, particulate enzyme with DIP; B, soluble enzyme with DIP; C, particulate enzyme with oxygen. The assays were otherwise carried out under standard conditions. The DIP reduction rates were corrected for decline in extinction with pH according to an empirical extinction curve with p*K* about 5.8.

pH-dependence

Fig. 1 shows the dependence on pH of the dehydrogenase reaction with DIP for the soluble and the particulate enzyme, and also of the aerobic reaction of the latter. There is a pronounced shift in the optimum when oxygen replaces DIP, indicating a still higher pH optimum for some reaction along the chain to oxygen, or a lower pH optimum for the dehydrogenation with DIP than with the natural primary acceptor.

TABLE IV

INHIBITION BY ATEBRIN

The measurements were carried out in the DIP-system under standard conditions, without preincubation of the enzymes with atebrin.

Atebrin concn. ($M \times 10^5$)	*Per cent inhibition*	
	Sol. enz.	*Part. enz.*
0.5	19	43
1.0	24	50
2.0	35	
4.4	52	64
8.8	68	
13.2	77	82

Inhibition studies

The soluble and particulate enzymes were inhibited by atebrin in the concentrations given in Table IV. The particle is slightly more sensitive to the inhibitor than the soluble dehydrogenase, but concentrations of 10^{-3} *M* or more are in both cases required to give 50% inhibition. *p*-Chloromercuribenzoate in a concentration of $3.6 \cdot 10^{-3}$ *M* did not interfere with the activity of the dehydrogenase, even after prolonged action. HCN was likewise found without effect on the dehydrogenase reaction in a concentration of 0.01 *M*. At this concentration, the aerobic oxidation with the particle was inhibited 20%. 0.001 *M* was, however, without effect on the oxygen uptake.

The involvement of cytochromes

Work by others on glucose oxidizing particles from microorganisms has demonstrated the reducibility of cytochromes of these particles by glucose. Such is also the case with the particles obtained from this organism. Fig. 2 gives difference spectra for the fraction of small particles, showing maxima at 427, 530 and 560 mμ. The particles, as one would expect, also carry other enzymes. DPNH dehydrogenase and oxidase activities were demonstrated, and found somewhat lower than the corresponding glucose activities, but of the same order of magnitude. The particles also posessed succinic dehydrogenase.

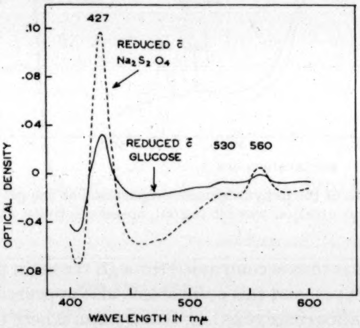

Fig. 2. Difference spectrum for small particle fraction. The particles were sedimented from a 45–55% ammonium sulphate fraction at 140,000 × *g* for 4 h. The protein concentration was 8.4 mg/ml, specific activity 0.5. Glucose and dithionite were added to a phosphate buffer suspension of the particles, and the absorption read against a cuvette with the same particle concentration.

These other dehydrogenase activities are absent from the purified dehydrogenase. Fig. 3 gives the spectral characteristics of the preparation which was the product of the particular purification reproduced in Table I. Before reduction, the preparations at this stage have maxima at 419 and 530 mμ. In some early preparations there was also a shoulder at 440–460 mμ, as is the case in Fig. 3. Upon addition of glucose to the cuvette, there is a more or less pronounced shift of the SORET band towards higher wavelengths and an appearance of bands around 530 and 560 mμ. These latter peaks are intensified by addition of dithionite, forming maxima at 532 and 562 mμ, while the SORET band shifts to 428 mμ. Preparations have been encountered where glucose alone causes this maximal reduction. For preparations with lower glucose–cytochrome reduction, one could instead of dithionite add traces of phenazin methosulphate, which, upon reduction by the dehydrogenase present, rapidly reduced the heme. DIP also acted as carrier in this fashion, but not as effectively as phenazine methosulphate.

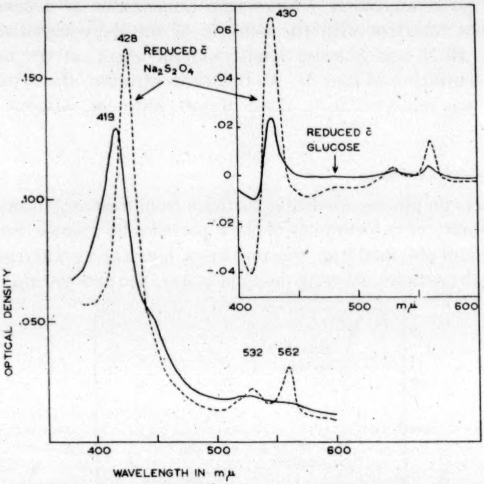

Fig. 3. Absorption spectrum of the dehydrogenase preparation at the gel eluate stage. The protein concentration was 1.6 mg/ml, specific activity 41.

The dehydrogenase is thus accompanied through the main purification process by a cytochrome of the *b*-types, and this cytochrome also appeared to have a functional relationship to the dehydrogenase reaction. It was natural here to first think of the enzyme as an analogue to yeast lactic dehydrogenase[1] or *Propionibacterium pentosaceum* succinic dehydrogenase[18]. There were suggestions of the presence of functional flavin (the shoulder at 440–460 mμ, the inhibition by atebrin). Purer preparations were, however, needed to decide this question of the possible role of flavin. The role of the heme was also somewhat obscure, not only in the variable glucose-reducibility, but also in the fact that the gel eluate fraction from different cell batches gave ratios of enzyme activity to heme content varying in a tenfold range, the highest specific activities corresponding to the lowest heme concentrations.

The further purification on carboxymethyl cellulose described above threw surprising light on some of these questions. Practically all the heme was eluted at pH values below 6.85, most of it appearing with the 0.03 *M* pH 6.60 buffer. The enzymic activity in these fractions was, however, negligible. The dehydrogenase appeared, as mentioned, with 0.05 *M* pH 6.85 buffer. Depending on the purity and heme content of the material applied to the column, the dehydrogenase fractions were either found very low in or essentially free from heme. Fig. 4 gives the spectral characteristics after concentration of pooled fractions with high heme/protein and activity/protein ratios respectively.

The material of Fig. 4, curve A, represents a 7-fold purification of the cytochrome *b* in the preparation applied to the column, a preparation similar in heme/protein/activity ratios to the preparation of Fig. 3. Based on an α-band extinction coefficient of $36 \cdot 10^6$ cm^2 × mole^{-1}, there is one mole of heme/290,000 g of protein. This coefficient was found by making the pyridin hemochromogen of a sample of cytochrome *b* similar to that of Fig. 4, and it is nearly the same as that of crystalline yeast lactic dehydrogenase[17]. The spectral maxima above 400 mμ are at the same wavelengths as noted for the preceding purification stage, and the pyridin hemochromogen showed maxima in the reduced state at 419, 525, and 557 mμ, confirming the assignment of the hemoprotein to the *b*-class of cytochromes, having protoheme as prosthetic group.

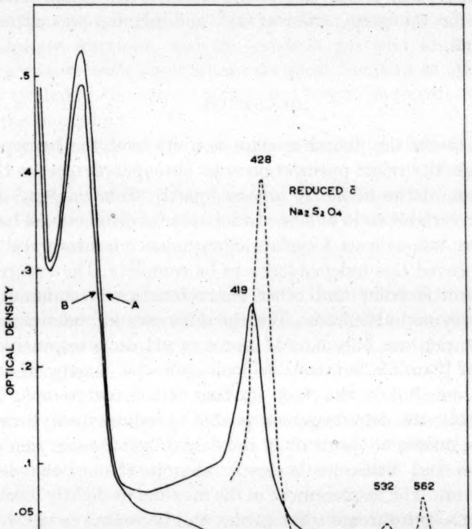

Fig. 4. Absorption spectrum of dehydrogenase and cytochrome *b* after carboxymethyl cellulose. A represents a concentrate of fractions 9–14 (0.03 *M* phosphate pH 6.60), B, fractions 21–23 (0.05 *M* phosphate pH 6.85), with specific activity 390. The actual O.D. obtained for A are here multiplied by 2.5, for the sake of comparison.

The material of Fig. 4, curve B, represents an 11-fold purification with respect to the dehydrogenase activity of the preparation applied to the column. Here there is only a small suggestion of heme absorption. The dehydrogenase spectrum further shows no indication of any flavin being present, and determination of the fluorescence of the preparation after digestion with trypsin likewise showed no sign of the characteristic flavin band.

The degree of purity of the final dehydrogenase preparations is not known. Too little material has so far been available to make the necessary homogeneity tests. The activities are, however, high. The preparation of Fig. 4 represents a 1,300-fold purification above the original extract, and it had a catalytic activity of 80 μmoles DIP reduced/min/mg under standard conditions. For excess glucose and DIP, the rate should ideally be 5-fold higher[19]. Because of mutual inhibition at high glucose and DIP concentrations, only 70% of this may be attained. This means that the maximal turnover-number for the preparation at 25° and pH 6.0 was 28,000 moles/min/100,000 g of protein.

DISCUSSION

An association between the dehydrogenase activity and the hemoprotein accompanying it through the main purification was anticipated, partly because of the observed reductions of the heme by glucose, partly from analogy considerations. Already the rather variable ratio of heme to activity for different cell batches showed, however, that there was at least a certain independence between the two. The final purification step proved this independence to be complete. The two proteins, which have a high isoelectric point and other characteristics in common, were finally separated on carboxymethyl cellulose. But the difference in buffer concentration and acidity for their elution was only 0.02 *M* and 0.25 pH units respectively.

It still appears plausible, however, to look upon the enzyme when operating in the cell, as cytochrome linked, the chain starting with cytochrome *b*. There are two main reasons for this: the dehydrogenase is able to reduce the cytochrome accompanying it, and the difference spectrum of the dehydrogenase-carrying particles upon addition of glucose and dithionite is closely akin to that of the dehydrogenase–cytochrome *b* mixture. The displacement of the maxima to slightly lower wavelengths could be due to some cytochrome *c*-like hemes also becoming reduced.

The first of these phenomena was, as already noted, rather variable, extending from full reduction to almost nil, independent of the dehydrogenase activity. Such variation in cytochrome reducibility has been observed by others, even with dehydrogenases where the heme still is a part of the enzyme[20]. Preliminary observations indicate that the level of cytochrome *b* reduction in the present work were expressions of steady states, where the reduction rate was equal to the slow autooxidation taking place. Factors decreasing the latter or increasing the former should then give a higher degree of observed heme reduction. The efficient transfer of electrons from the substrate to cytochrome *b* could possibly *in vivo* involve a cellular equivalent to the artificial bridge afforded by phenazine methosulphate in our experiments. This natural carrier might to a large degree be lost during the purification. Flavin does apparently not play this role, as there is no reduction by the dehydrogenase of either FAD or FMN. An efficient *in vivo* transfer may, however, alternatively be brought about by a close proximity of dehydrogenase and cytochrome *b* in the cell structure.

The argument from the observations on the spectral properties of the particles rests on the assumption that the particle is an expression of how the dehydrogenase is fitted into a larger structure in the cell. The similarity in acceptor specificity, in pH dependence, and in sensitivity towards inhibitors, together with the fact that both forms lead to a one-step oxidation of glucose, supports this view. The substrate specificity data given in the accompanying paper[19] also support this contention, if one is willing to grant that the incorporation of the dehydrogenase into, or separation from, the particles may modify the architecture of the substrate site to a certain degree.

The enzyme and cytochrome-carrying particles discussed are probably to be considered as artifacts produced in the grinding. There exist in the homogenate many sizes of particles carrying the ability to oxidize glucose, some of which are sedimented in the clarification centrifugation, some in the protamin sulphate and 0–45 % ammonium sulphate fractions, and the smallest particles finally in the 45–55 % fraction. In accordance with observations on ghost fractions in other organisms[21, 22], these particles probably represent smaller and larger fragments of the cytoplasmic membrane of the bacterium.

Microbial cytochromes of the *b*-group are usually classified into three classes, cytochrome *b*, cytochrome b_1 and cytochrome b_2. PAPPENHEIMER[23] differentiates according to the position of the α-band, so that cytochromes with maxima at 562–565, 560, and 556 mμ should be designated with *b*, b_1, and b_2 respectively. According to this convention, the cytochrome here purified should be classified as a cytochrome *b* without subscript.

Few microbial *b*-type cytochromes have been purified in the soluble state. VERNON[24] has isolated soluble cytochrome b_1 from *Micrococcus lysodeikticus* and *Pseudomonas denitrificans*, showing maxima at 426, 528, and 559 mμ. A soluble form of succinic dehydrogenase from *Propionibacterium pentosaceum*[18] has also been somewhat purified, showing maxima around 430, 530, and 560 mμ. Cytochrome b_2 is best represented, with the essentially homogeneous or crystalline lactic dehydrogenase preparations from yeast[17, 20]. These have equivalent weights of about 100,000, as compared to 290,000 for the present preparation, which probably still is inhomogeneous.

The *Bacterium anitratum* glucose dehydrogenase functions without addition of niacinamid coenzymes, and does not reduce these. If the enzyme, as postulated, stands at the entry of the cytochrome system, it is perhaps even more noteworthy that flavin is not involved. The inhibition by relatively high concentrations of atebrin observed for the present enzyme is without relation to flavin function. Instead, it could be shown that atebrin competes with the acceptor DIP for a site on the enzyme[19].

As already mentioned, it can not be ruled out that another unidentified compound acts as a carrier *in vivo*, reducing cytochrome *b* after having itself become reduced, and a flavoprotein might conceivably play this role. But whether DIP in the present assay is to be regarded as a substitute for cytochrome *b*, or for an intervening carrier, there remains the question whether the dehydrogenase "itself" becomes reduced in a particular group, or whether there is a direct transfer of the hydrogen from substrate to acceptor. As is discussed elsewhere[19], the kinetic data with glucose and DIP are in line with the concept of a reducible group tightly bound to the enzyme taking part in the reaction, and do not show the characteristics of the usual direct reduction-oxidation reactions. This might possibly be fortuitous, and further chemical evidence is required to settle the question of mechanism.

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KINETICS AND SPECIFICITY OF GLUCOSE DEHYDROGENASE FROM *BACTERIUM ANITRATUM*

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SUMMARY

Soluble glucose dehydrogenase from *Bacterium anitratum* acts on a number of aldose sugars, with decreasing rate as the structure is removed from that of D-glucose.

Linear double reciprocal plots were obtained for glucose with a series of acceptor (2,6-dichlorophenolindophenol) concentrations, and these were in the main parallel. The independence of the slope on the acceptor concentration suggests a mechanism involving a reducible group on the enzyme.

Deviation from parallel linearity at high concentrations of substrate and acceptor could be interpreted as mutual competitive inhibition between substrate and acceptor. Atebrin was shown to inhibit through competition with the acceptor.

MICHAELIS constants, maximal velocities, and inhibition constants for the reaction are presented and discussed in terms of the mechanism proposed.

METHODS AND MATERIALS

The enzyme preparations used were prepared as described in the preceding paper[1]. The initial reaction rates were determined in 0.05 M phosphate buffer of pH 6.0 in a thermostated Beckman DU at 25°. All velocity data given are per mg protein.

The carbohydrates and other substances tested were obtained as indicated earlier, and from the following firms: D-xylose, L-xylose, D-ribose, D-fructose and D-galactose from Hoffmann-La Roche & Co; L-arabinose from Merck AG; D-arabinose from Nutritional Biochemicals Corporation; D-glucose-6-phosphate from Sigma Chemical Company; DL-glyceraldehyde from California Foundation for Biochemical Research. D-2-desoxyglucose was a gift from Dr. O. WALAAS.

RESULTS AND DISCUSSION

Specificity

The relative reaction rates with 0.02 M solutions of some aldoses and $4.0 \cdot 10^{-5}$ M DIP are given in Table I. In addition to these compounds, D-fructose, D-glucose-6-

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Abbreviation: DIP, 2,6-dichlorophenolindophenol.

phosphate, sorbitol, and mannitol were tested and found not to be attacked. Within the aldoses, the dehydrogenase exhibits a rather wide, but characteristic, specificity pattern. Based on the limited number of substances tested, it appears that in order to have a relative activity above 50 %, the configuration must start with (+ —).

TABLE I

SPECIFICITY

Substrate	*Configuration*	*% of maximal activity*	
		Sol. enz.	*Part. enz.*
D-glucose	+ — + +	100	100
D-galactose	+ — — +	66	
L-arabinose	+ — —	51	100
D-mannose	— — + +	30	
D-xylose	+ — +	27	100
D-ribose	— — —	7.5	
D-2-desoxyglucose	0 — + +	4.5	
D-arabinose	— + +	1.6	1.5
DL-glyceraldehyde	+ —, — +	1.0	
L-xylose	— + —	0	0

It is interesting to note that for the pentoses tested with the soluble enzyme, it is more important that this (+ —) is followed by a (—) as in L-arabinose, than by a (+), as in D-xylose, although the latter appears more akin to D-glucose in structure. The pattern for the soluble and the particulate enzyme is the same in that inversion of the first two hydroxyls to (— +) leads to virtual or complete disappearance of activity. The particle, however, fails to distinguish between D-glucose, L-arabinose, and D-xylose under the conditions used. One is therefore led to pursue the specificity relationships further in terms of MICHAELIS constants and maximal velocities.

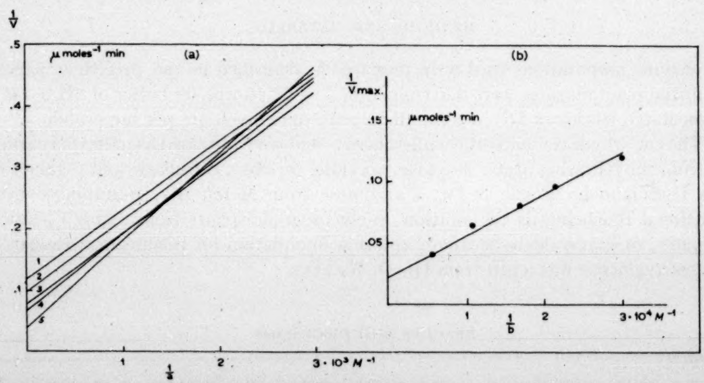

Fig. 1. Dependence of reaction rate on substrate and acceptor concentrations. 1a: Lines 1–5 are reproductions of double reciprocal plots determined by five glucose concentrations from 3.3 to $200 \cdot 10^{-4}$ *M*. The DIP concentrations were 3.3, 4.6, 5.8, 9.2 and $18.2 \cdot 10^{-5}$ *M* respectively. 1b: The intercepts on the $1/V$ axis of Fig. 1a are here plotted against $1/b$. The enzyme preparation used had a specific activity of 44 units/mg.

Kinetics of the glucose-DIP reaction

The initial rate of reaction was determined for a series of DIP concentrations, each with a series of glucose concentrations. In double reciprocal plots a system of straight lines appeared. The lines are reproduced in Fig. 1a. Fig. 1b is a plot of the reciprocal maximal velocities thus obtained against the inverse DIP concentrations. The linear relationships obtained show that the data conform to the two-substrate rate equation of DALZIEL[2]

$$\frac{(E)_0}{V} = \phi_0 + \frac{\phi_1}{a} + \frac{\phi_2}{b} + \frac{\phi_{12}}{ab} \tag{1}$$

where a and b in the present case are the initial concentrations of glucose and DIP respectively, and $(E)_0$ is the concentration of active enzyme sites.

ϕ_{12} is zero for DIP concentrations in the range 1.0 to $6.0 \cdot 10^{-5}\ M$, as shown by the fact that the $1/V$ *versus* $1/a$ lines are parallel (Fig. 1a, 2a). The parameter may well be zero in the whole range measured, which extends to $18 \cdot 10^{-5}\ M$. The slope in this higher region increases, rather than decreases, as would be expected if ϕ_{12} was not zero. This change in slope is due to an inhibition by DIP. In addition to this inhibition, revealing itself particularly at high DIP and low glucose concentrations, one also observes (Fig. 2) an inhibition for high glucose and low DIP concentrations. If these inhibitions are competitive, as is indicated by the nature of the curves, they may be taken into account in the equation by multiplying the ϕ_1 term with $(1 + b/K_{ib})$, and the ϕ_2 term with $(1 + a/K_{ia})$, K_{ia} and K_{ib} being inhibition constants (Eqn. (2)). These factors are essentially unity for a large enough portion of the data of Fig. 1 to allow $1/(E)_0 \times \phi_0$, ϕ_1, and ϕ_2 to be computed.

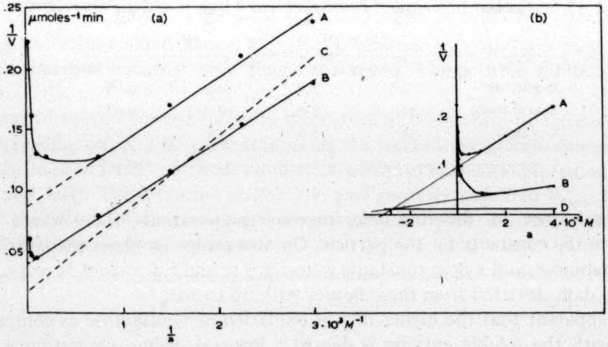

Fig. 2. Inhibition by excess substrate and acceptor. 2a: Curve A and B correspond to DIP concentrations of 1.0 and $4.6 \cdot 10^{-5}\ M$ respectively. The enzyme preparation had a specific activity of 108 units/mg. Line C has been transposed from Fig. 1a, where it had number 5. 2b: $1/V$ is here for the same experiment replotted against the substrate concentration. Line D represents the ideal velocity for excess substrate and acceptor, 112 µmoles × min⁻¹ × mg⁻¹.

From the slope of the parallel lines of Fig. 1a, we find $\phi_1/(E)_0 = 1.16 \cdot 10^{-4}$ µmoles⁻¹ × min × M. From the slope of Fig. 1b: $\phi_2/(E)_0 = 3.4 \cdot 10^{-6}$ µmoles⁻¹ ×

min × M, and from the intercept of the same figure: $\phi_0/(E)_0 = 2.2 \cdot 10^{-2}$ µmoles^{-1} × min. The MICHAELIS constant for glucose, DIP in excess, was $\phi_1/\phi_0 = 5.3 \cdot 10^{-3}\,M$, the corresponding constant for DIP $\phi_2/\phi_0 = 1.6 \cdot 10^{-4}\,M$, and the maximal velocity per mg of this particular dehydrogenase preparation $(E)_0/\phi_0 = 45$ µmoles × min^{-1}, as compared to 400 µmoles × min^{-1} × mg^{-1} for the most active preparation isolated so far. These MICHAELIS constants and maximal velocities are ideal ones, as the inhibitions have not been taken into account.

Specificity analysis

Apparent MICHAELIS constants ($K_M^{\text{ap}} = \phi_1/(\phi_0 + \phi_2/b)$) and maximal velocities ($V_{\text{max}}^{\text{ap}} = (E)_0/(\phi_0 + \phi_2/b)$) obtained with L-arabinose, D-xylose, and D-glucose for the soluble and the particulate enzyme are compared in Table II. Whereas the various MICHAELIS constants for the soluble enzyme have been found identical, within experimental error, for differing dehydrogenase preparations, there was a certain variation in the constants for the particle. On an average for three preparations,the glucose, arabinose, and xylose constants were 1.5, 7.6, and 2.9 × $10^{-4}\,M$ respectively. Individual data deviated from these figures with up to 50%.

TABLE II

SPECIFICITY CONSTANTS

A soluble enzyme preparation with specific activity of 31 units/mg was used, and the particulate form had 0.6 units/mg. DIP was present in a concentration of $4.0 \cdot 10^{-5}\,M$. V_{max}-values are given as µmoles × mg^{-1} × min^{-1}.

Substrate	*Soluble enzyme*		*Particulate enzyme*	
	K_M^{ap} $(M \times 10^3)$	V_{max}^{ap}	K_M^{ap} $(M \times 10^4)$	V_{max}^{ap}
D-glucose	1.1	7.0	2.3	0.08
L-arabinose	22	7.0	10.0	0.08
D-xylose	51	7.0	3.0	0.08

It is apparent that the higher rate of oxidation of L-arabinose as compared to D-xylose with the soluble enzyme is due to a lower ϕ_1-value, suggesting a higher affinity of the enzyme for this substrate, and not to a difference in reaction rate once saturation has been assured. Identical $V_{\text{max}}^{\text{ap}}$-values were also obtained with the particulate enzyme for all three substrates. The K_M^{ap}-values have, however, been strikingly reduced, and not to the same degree. D-xylose here appears as a better substrate than L-arabinose, and as only little inferior to D-glucose. An approximate determination of ϕ_2/ϕ_0 showed that this ratio in the particulate form was five times lower than for the soluble enzyme. There has thus been a relatively larger increase in ϕ_0 than in ϕ_2. ϕ_1 has in addition been affected in a specific manner not eliminated by division with ϕ_0, as shown by the changes in K_M^{ap} for the substrates.

Table II serves to emphasize the dependence of specificity data on the substrate concentration used. Had the data of Table I been collected at a lower substrate level, the relative preference for glucose would have appeared greater.

Inhibitions

From the data given in Fig. 2 approximate values for the inhibition constants K_{ia} and K_{ib} referred to above may be calculated. The positions of the $1/V$ *versus* $1/a$ lines for the highest DIP concentrations in relation to the parallel lines obtained at lower concentrations are such (Fig. 1a, 2a) as would be expected for a competitive inhibition. The ratio of the slopes for lines C and B in Fig. 2a is 1.25. Equating this with $(1 + b/K_{ib})$, which is valid both for competitive and noncompetitive inhibition, a value of $7 \cdot 10^{-4}\ M$ for K_{ib} is obtained. The inhibition constant is thus 7-fold lower than the glucose MICHAELIS constant given above.

In order to obtain K_{ia}, the points on curve A and B in Fig. 2a were, following DIXONS method[3], replotted as $1/V$ *versus* a (Fig. 2b). The extrapolated straight line sections cross each other at a $1/V$ value in good agreement with the expected $1/V_{\max}$, and at a negative glucose concentration of $2.8 \cdot 10^{-2}\ M$, directly giving K_{ia}.

Because of these inhibitions, the maximal velocity $(E)_0/\phi_0$ can not be attained. Analysis of Eqn. (2)

$$\frac{(E)_0}{V} = \phi_0 + \frac{\phi_1}{a}\left[1 + \frac{b}{K_{ib}}\right] + \frac{\phi_2}{b}\left[1 + \frac{a}{K_{ia}}\right] \tag{2}$$

reveals that $(E)_0/V\phi_0$ asymptotically approaches $V_{\max}/V'_{\max} = 1 + 2\sqrt{\dfrac{\phi_1\phi_2}{\phi_0^2 K_{ia}K_{ib}}}$ as its minimal value when a and b are increased in the optimal ratio $a/b = \sqrt{\dfrac{\phi_1 K_{ia}}{\phi_2 K_{ib}}}$ Inserting the values found above, $V_{\max}/V'_{\max}$ becomes 1.42, and a/b 36.5. 70 % of the ideal maximal velocity may thus be reached, competitive inhibition being assumed.

It was of special interest to study the nature of the atebrin inhibition reported in the preceding paper. Fig. 3 gives the result of a test of three atebrin concentrations with three levels of DIP. The distribution of the points indicates a competition between atebrin and DIP. The intercept on the $1/V$ axis gives the expected $V_{\max}$. From the ratio of the slopes of the two top lines to the lower one, K_i-values of 3.8 and $4.0 \cdot 10^{-3}\ M$ respectively are obtained.

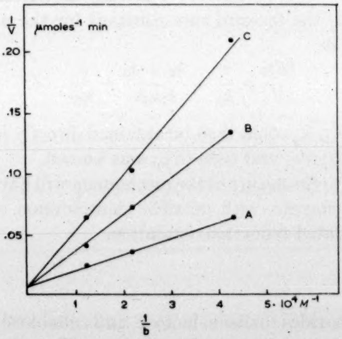

Fig. 3. Competitive inhibition by atebrin. The concentrations of atebrin were for the three series A, B, and C: 0, 4.4, and $8.8 \cdot 10^{-3}\ M$ respectively. The enzyme preparation was the same as in Fig. 2.

DISCUSSION OF MECHANISM

It is hasardous to suggest a mechanism for the reaction on the basis of the data presented. If one makes the assumption, however, that ϕ_{12} really is zero, and not merely too small to be detected, one is directed to the type of mechanism where the enzyme is first reduced by the substrate and subsequently reoxidized by the acceptor. Among the enzyme reactions of the general form $A + B \rightleftharpoons C + D$ discussed by ALBERTY[4, 5], this type is the only one which fulfils the criteria of $S_{12} = 0$; $\phi_0, \phi_1, \phi_2 \neq 0$. It may be formulated as follows:

$$SH_2 + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SH_2E \overset{k_3}{\rightleftharpoons} S + EH_2 \tag{3}$$

$$EH_2 + A \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} EH_2A \overset{k'_3}{\rightleftharpoons} E + AH_2 \tag{4}$$

Initial steady state treatment of this set of reactions leads, as shown earlier[4,6], to the following expression:

$$\frac{(E)_0}{V} = \frac{1}{k_3} + \frac{1}{k'_3} + \frac{k_2 + k_3}{k_1k_3a} + \frac{k'_2 + k'_3}{k'_1k'_3b} \tag{5}$$

This is of the same form as the empirical rate expression (1) above, with $\phi_{12} = 0$ as the characteristic feature.

The change noted in the ϕ_2/ϕ_0 ratio in going from the soluble to the particulate enzyme could be explained by a decrease in k'_3 alone, permitting the constants of the first step (3) to be the same in the two forms of the enzyme. The variable reductions in ϕ_1 observed show, however, that also the latter constants are affected. If the "unitary" hypothesis[1] is to be retained, it must be admitted that the separation of the primary dehydrogenase from the particle, or the incorporation of it into the particle, has resulted in some modifications of the place of substrate attachment, as well as of the reoxidation mechanism.

If one assumes no connection to exist between the sets of data for the soluble and the particulate enzyme, it is possible to write a simpler mechanism that still conforms to the observed rate law. This is done by omitting the MICHAELIS complex in the second step (4). Denoting the forward rate constant for the new second step k_4, the new rate expression will be

$$\frac{(E)_0}{V} = \frac{1}{k_3} + \frac{k_2 + k_3}{k_1k_3a} + \frac{1}{k_4b} \tag{6}$$

k_3, k_4, and $K_M = (k_2 + k_3)/k_1$ could then be obtained directly from the ϕ-parameters, once the purity of the enzyme, and thus $(E)_0$, was known.

Further evidence as to the nature of the mechanism will have to be sought through chemical studies of the enzyme, with possible identification of a group that can be responsible for the postulated reduction–oxidation.

NOTE ADDED IN PROOF

The reducing disaccharides maltose, lactose, and cellobiose were attacked almost as readily by the soluble enzyme as D-glucose, their K_m values being 4.7, 3.3, and $2.2 \cdot 10^{-3}$ M. With melibiose, a K_m of approximately 0.2 M was found.

The data reported in the paper refer to mutarotational equilibrium mixtures. For glucose it could be shown that the β-anomer is preferentially attacked: when the reaction was started by the addition of 10 μl of a freshly prepared cold 0.1 *M* solution of glucose, the reaction rate in the first half minute was only 10 % of that observed after the reaction had proceeded at 25° for 6 min.

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STIMULATION OF AMYLASE FORMATION BY AN AMINE FROM *BACILLUS SUBTILIS*

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SUMMARY

An active factor which stimulates amylase formation by *Bacillus subtilis* has been extracted from *B. subtilis* cells and purified. The factor resembles alkyldiamines or polyalkylamines in its chromatographic behavior, its reaction with ribonucleic acids and other types of reactions.

Authentic samples of amines such as putrescine, cadaverine, spermidine and spermine have been found to stimulate amylase formation at high concentrations. None of them however is identical with the factor.

The factor also stimulates the production of other exo-enzymes of *B. subtilis*, *e.g.*, proteases, ribonuclease, and γ-glutamyltransferase. Moreover it stimulates the turnover of RNA and polyphosphate in the cells to a greater extent than it stimulates enzyme production.

In vitro experiments showed that in the presence of the factor, RNA associates to larger molecules than those of the original RNA.

The chemical structure of the factor and its biological functions are discussed.

INTRODUCTION

It has been reported previously that the formation of amylase from resting cells of *Bacillus subtilis* was stimulated by a ribonucleic acid (RNA) fraction prepared from cells of the same strain[1].

Treatment of the RNA fraction with ribonuclease (RNase) did not abolish the stimulating activity, and the acid soluble fraction of the RNase digest (R-Hps) was used as an active crude factor. The activity of this factor was destroyed by acid or alkaline hydrolysis, and preparations obtained from the cells which produce small amount of amylase were also inactive. From these experimental results, the active moiety of the factor was presumed to be either a specific oligonucleotide or a specific substance attached to nucleic acid.

Further fractionation of R-Hps was carried out. During the purification of the factor extensive elimination of RNA had no effect on the activity of the factor and an almost pure active material was isolated. This material was basic and had a strong affinity for RNA.

There is some evidence that basic materials, *i.e.*, basic amino acids or biological amines[2, 3], may play some essential role in the protein synthesis especially by interaction with acidic materials such as RNA and deoxyribonucleic acid (DNA).

In the present paper the procedure for purification, the chemical nature of the purified material, and some of its biological functions are presented. The data obtained suggest that the active moiety is a derivative of an alkyldiamine or a polyalkylamine.

METHODS

Strain

Bacillus subtilis strains, H, N, and K were used in this investigation. Strain H was mainly used as a source of active factor and strain N was used for the assay of the active factor.

Media and culture

The media and the cultures of the cells used both for the preparation of the factor and its assay have already been reported in an earlier paper[1]. The preparation of an assay system for the active moiety has been also described previously[1].

Assay procedure

Amylase was assayed by the method of HAGIHARA[4]. Protease was assayed by the method of CHARNEY AND THOMARELL[5]. RNase was assayed by the method of REDDI[6]. γ-glutamyltransferase was determined by measuring the optical density of the color which is formed on reaction of γ-L-glutamylhydroxamate with $FeCl_2$ at 540 mμ in a Beckman-type spectrophotometer.

Analytical methods

Protein and RNA were fractionated by the method of SCHMIDT-TANNHAUSER[7]. The total nucleic acid content was measured from the u.v. absorption, assuming that the optical density at 260 mμ of nucleic acid solution containing 1 mg/ml is 22 [8]. The protein content was determined by FOLIN's reagent[9]. Inorganic phosphate was determined by ALLEN's reagent[10]. The radioactivity was determined by a conventional Geiger-Müller counter with an end window.

RESULTS

Purification of the active factor

The preparation of R-Hps has already been reported[1]. The procedure for the further purification of the active factor is presented in Fig. 1, and the analytical data obtained in different stages of the purification are shown in Table I.

When RHps was added at pH 7.3 onto the Dowex-1 (Cl) column and eluted with water, more than 90 % of the biological activity was eluted out, although 95 % of the u.v. light absorbing materials contained in the RHps preparation remained in the column. It is clear, therefore, that extensive elimination of the ribonucleic acid components had no effect on the activity of the factor. The non-RNA nature of the active material is very similar to that of the incorporation factor described recently by GALE AND FOLKES[11].

Activated charcoal was added to the eluate from Dowex-1 at 20 mg/ml which was then filtered through sintered glass after stirring for 15 min. Approximately 50 %

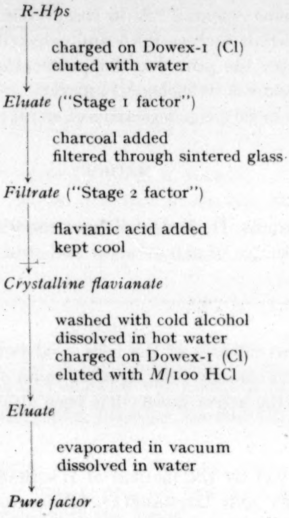
R-Hps
charged on Dowex-1 (Cl)
eluted with water
Eluate ("Stage 1 factor")
charcoal added
filtered through sintered glass
Filtrate ("Stage 2 factor")
flavianic acid added
kept cool
Crystalline flavianate
washed with cold alcohol
dissolved in hot water
charged on Dowex-1 (Cl)
eluted with *M*/100 HCl
Eluate
evaporated in vacuum
dissolved in water
Pure factor

Fig. 1. Fractionation diagram for active factor.

TABLE I

ANALYTICAL DATA FOR VARIOUS FACTOR PREPARATIONS

Various factor preparations derived from the same amount of original preparation, Hp, which contained 1 mg/ml RNA were analysed. The results of the analysis of similar samples were in agreement with the data presented here.

Stages of purification	*RNA (mg/ml)*	*Protein (mg/ml)*	*Ninhydrin value* (mg/ml)*	*Polysaccharide** (mg/ml)*	*Dry weight (mg/ml)*
Hp	1.0	1.3	0.18	0.38	
R-Hps	0.63	0.18	0.13	0.05	1.9
Stage-1 factor	0.028		0.096	0.035	1.0
Stage-2 factor	0.00	0.00	0.064	0.00	0.3
Pure factor	0.00	0.00	0.016	0.00	0.05

* The amounts are shown as leucine equivalents.

** Polysaccharide is measured by anthron reagent and is shown as glucose equivalents.

of the total activity was recovered in the filtrate. By this procedure the remaining nucleic acid components and oligosaccharides were exclusively eliminated. Paper-chromatography of the charcoal-treated factor preparation (stage 2 factor) was carried out. The charcoal filtrate was concentrated and chromatographed on Toyoroshi filter paper No. 51, 10 × 40 cm, in the three solvent systems shown in Table II. In each system a single spot was obtained which reacted with ninhydrin reagent. After the chromatograms had been developed and dried, they were cut into pieces and were eluted with water to test for biological activity. Both the active fraction and the ninhydrin positive spot were found in the same region. Thus it is supposed that the material which is reactive with ninhydrin is the active moiety. By paper electrophoresis it was shown that the active material was positively charged at pH 6.5 (*cf.* Table II).

Further purification was attempted by precipitation with flavianic acid when yellow needle-like crystals were easily obtained from cold aqueous solution. The crystals were washed twice with cold absolute alcohol and dissolved in hot water. The solution was then charged on Dowex-1 (Cl) column and eluted with $M/100$ HCl. About 30% of the ninhydrin positive material originally contained in the stage 2 factor was eluted and the flavianic acid remained on the column. After the HCl had been evaporated in a vacuum the residue was shown to stimulate amylase formation.

TABLE II

CHEMICAL NATURE OF THE FACTOR: COMPARISON WITH SOME AMINES

Factor and amines	*RF on paper chromatography*[*]			*Mobility on paper electrophoresis*[**] *(cm)*	*Biological activity*[***] *(% activation)*
	Solvent I	*Solvent II*	*Solvent III*		
Stage 2 factor	0.05–0.15	0.42–0.50	0.51–0.57	—3.8 – —4.8	390
Putrescine	0.08–0.12		0.57–0.60	—8.3	50
Cadaverine	0.12–0.18	0.30–0.50	0.60–0.63	—8.0	135
Spermidine	0.00–0.04		0.32–0.40	—4.8	151
Spermine	0.00–0.08	0.28–0.60	0.17–0.23	—4.8	240

[*] All the paper chromatography was carried out by the ascending method at room temperature. Solvent I: butanol–acetic acid–water = 4:1:1 (by volume); Solvent II: butanol saturated with concentrated NH_4OH; Solvent III: methylcellosolve–propionic acid–water = 75:15:15 (by volume).

[**] Conditions of electrophoresis: Toyoroshi No. 51, 2 × 15 cm, $M/10$ citric acid buffer, pH 6.5, 200 V, 2.7 mA, for 2 h.

[***] Biological activity was assayed in resting cells.

Chemical nature of the factor

As the yield of the pure factor is very small, stage 2 factor is used throughout the following experiments.

The factor showed a positive ninhydrin reaction but the color reactions for hexoses, ribose, hexoseamine, aromatic amines, histidine, and arginine were all negative. The factor is basic and combines with inorganic phosphate, flavianic acid and ribonucleic acid (*cf.* Fig. 3).

It was shown in Table II that the factor has the properties of polyalkylamines or polymethylenediamines judging from its behavior on both paper chromatography and paper electrophoresis using the appropriate solvent systems of HERBST *et al.*[12]. We have not yet found the natural amine which is identical with the factor.

Effect of alkyldiamines and polyalkylamines on amylase formation

As shown in Table II some natural amines, putrescine, cadaverine, spermidine, and spermine, showed a stimulating effect on amylase formation: spermine has the highest activity, almost 2/3 of that of the purified factor from *B. subtilis*. The concentration of the amines which is necessary to show their activity, however is five or ten times higher than that of the factor. The saturation curve of the effect of the factor on amylase and protease is presented in Fig. 2. These results strongly support the idea that the active material is a polyalkylamine, alkyldiamine, or one of their derivatives.

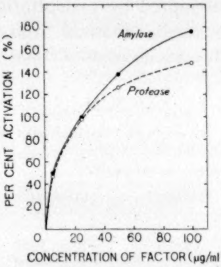

Fig. 2. Saturation curve of the effect of factor on amylase and protease formation.

$$\text{Percent activation} = \frac{(\Delta\,\text{enzyme activity with addition} - \Delta\,\text{enzyme activity without addition}) \times 100.}{\Delta\,\text{enzyme activity without addition.}}$$

Resting cells were incubated for 3 h. with the addition of various amounts of the factor, and the increase of enzyme activity was measured.

Biological functions of the factor

Stimulation of enzyme formation: Resting cells of *B. subtilis* strain N form exoenzymes, protease, and ribonuclease together with amylase[1, 13, 14]. Also resting cells of strain K form the exoenzyme γ-glutamyltransferase. As shown in Table III the formation of these exoenzymes is stimulated by the factor. NISHIMURA found that strain N has ribonuclease inside the cell which is different in various respects from the exoenzyme ribonuclease[15]. In resting cells, however, neither an increase of internal ribonuclease nor the effect of the factor on the formation of the enzyme was observed.

Reaction of factor with RNA in vitro: As mentioned above, the factor gives an insoluble complex with inorganic phosphate in water. It was also shown that the factor combined with RNA. Commercial yeast RNA was dissolved (1 mg/ml) in $M/30$ tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.3). When the RNA solution was added to two volumes of 99.5 % alcohol in ice water, precipitation of RNA was

TABLE III

THE EFFECT OF THE FACTOR ON THE FORMATION OF EXOENZYMES

The amounts of amylase and protease were calculated by comparing the activities of the enzymes in the culture aliquots with the activities of the crystalline enzymes. Ribonuclease was calculated by comparing with purified ribonuclease. γ-glutamyltransferase was determined by measuring the optical density at 540 mμ of the color of γ-glutamylhydroxamate assuming that a 1.0 increase in O.D.^{540} was 1 unit.

Enzymes	*Increase of enzyme activity/3 h (μg/ml)*		*Per cent activation (%)*
	No addition	*+ Factor*	
Amylase	17	45	170
Protease	8	32	300
Ribonuclease	0.04	0.12	200
γ-Glutamyl transferase	0.2	0.55	170

not observed. But when alcohol was added to the RNA solution in the presence of low concentrations of the factor, heavy precipitation occurred at once. Alcoholic precipitation of 0.1 % RNA solution in the presence of various amounts of the factor, cadaverine, and spermine were examined and the results are presented in Fig. 3 and Table IV. It is shown that the factor as well as cadaverine and spermine at low concentrations combined with RNA to produce complexes insoluble in 66 % alcohol. The factor has the strongest affinity for RNA.

TABLE IV

PRECIPITATION OF RNA IN THE PRESENCE OF THE FACTOR

The experimental method and the reaction mixture are shown in Fig. 3. The amount of the factor soluble in 66 % alcohol was measured with ninhydrin and calculated in leucine equivalents. The amount of the factor shown in the last column was calculated by the difference between the amount of the factor added and that which was soluble in 66 % alcohol.

Amounts of the factor added (μg)	*Per cent of RNA soluble in 66 % alcohol* (%)	*Amounts of the factor soluble in 66 % alcohol* (μg)	*Amounts of the factor precipitated with RNA* (μg)
0	100	0	0
7.5	102		
37.5	7	< 5	> 32.5
75	2	< 5	> 70
375	2	266	109
750	2	578	172

A change of molecular weight of RNA in the solution in the presence of the factor was assumed and the sedimentation velocity of the RNA solution (5 mg/ml) was determined by an analytical Spinco E type ultracentrifuge in the presence and absence of the factor (450 μg/ml). The results showed that the sedimentation constant of RNA at 20° is 2.7 in the absence of the factor and is 4.2 in the presence of the factor. This means that the size of the molecule of RNA is, in the presence of the factor, approximately twice the size of the original one.

Effect of the factor on the metabolism of RNA in the cell: The effect of the factor on the incorporation of ^{32}P-labeled inorganic phosphate into RNA in the cell was examined.

Resting cells of *B. subtilis* strain N suspended in *M*/30 phosphate buffer (pH 7.3) were shaken at 30° for 60 min, and then ^{32}P-labeled inorganic phosphate was added. Aliquots were taken at various times and a RNA fraction was separated by the method of SCHMIDT-TANNHAUSER[7].

The RNA content of the cell decreased gradually during the incubation and after 60 min it was 70–80 % of the original amount. The rate of incorporation of ^{32}P into RNA is very slow. It was seen that after 60 min less than 1 % of the total phosphate in the RNA was labeled with ^{32}P.

As shown in Fig. 4 the factor stimulated the incorporation reaction. Both the incorporation of ^{32}P into control RNA and the stimulation of the factor were inhibited by the addition of 2,4-dinitrophenol ($1 \cdot 10^{-4}$ *M*). In many experiments the stimulating effect of the factor on ^{32}P incorporation runs parallel with that on enzyme formation.

But the former is always larger (5–10 times stimulation) than the latter (2–3 times stimulation).

Effect of the factor on the incorporation of ^{32}P into polyphosphate. Preliminary studies by paper chromatography showed that the greater part of ^{32}P incorporated into the RNA fraction is in phosphate compounds other than the four nucleotides. These unknown phosphate compounds are supposed to be polyphosphates. Therefore, the cells were incubated with ^{32}P-labeled inorganic phosphate for 2 h and fractionated

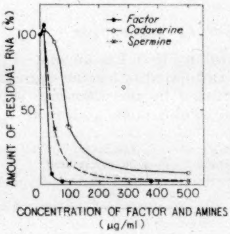

Fig. 3. Precipitation of RNA in the presence of factor and amines. 1 mg yeast RNA was dissolved with various amounts of factor and amines in 1 ml $M/30$ Tris buffer (pH 7.3). After incubation at 0° for 1 h 2 volumes of 99.5% alcohol were added and the precipitate centrifuged. The RAN content in the supernatant fraction was measured.

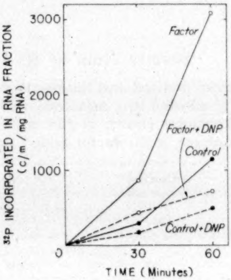

Fig. 4. Incorporation of ^{32}P-labeled inorganic phosphate into RNA fraction. Incubation mixture: washed cells suspended in $M/15$ phosphate buffer (pH 7.3), 1.5 ml; addition of ^{32}P-labeled inorganic phosphate, $6.5 \cdot 10^5$ counts/ml; 2,4-dinitrophenol, $1 \cdot 10^{-4}$ M; factor, 100 μg/ml as leucine equivalents; total volume = 3 ml.

TABLE V

THE INCORPORATION OF ^{32}P-LABELED INORGANIC PHOSPHATE INTO RNA AND POLYPHOSPHATE FRACTIONS

The methods of incubation and fractionation of RNA and polyphosphate are described in the text. Incubation mixture: washed cells suspended in $M/15$ phosphate buffer (pH 7.3), 1.5 ml; addition of ^{32}P, $5 \cdot 10^6$ counts/ml; factor, 100 μg/ml; total volume 3 ml.

	Time 0 h	*After 2 h*		*Per cent activation (%)*
		Control	*+ Factor*	
Amylase activity (μ/ml)	0.6	1.1	2.4	260
RNA fraction				
Amounts of RNA (mg/ml)	0.27	0.11	0.11	
Total counts (counts/ml)	20.4	270	1070	
Specific activity (counts/mg RNA)	76	2450	9700	296
Polyphosphate fraction				
Amounts of polyphosphate (mg/ml)	0.013	0.015	0.015	
Total counts (counts/ml)	451	3430	27000	
Specific activity (counts/μg poly-p)	34.6	228	1800	690
$\frac{\text{poly-p}}{\text{RNA + poly-p}} \times 100$*	95.8	92.7	95.2	

* The ratio of total counts of the fractions.

as follows: the cells were washed with cold 5 % perchloric acid (PCA) and then washed with a hot ethanol–ether mixture. The insoluble fraction was then extracted with 1 *M* NaCl for 60 min in a boiling water bath. RNA was precipitated with alcohol from the NaCl extract. The remaining fraction, insoluble in hot NaCl, was extracted with 5 % trichloroacetic acid for 15 min in boiling water. The extracted fraction contained a small amount of u.v. light absorbing material, and a large amount of inorganic phosphate was released after hydrolysis with 1 *M* HCl for 10 min in boiling water. This fraction represented the polyphosphate fraction.

The incorporation of ^{32}P into both the RNA and polyphosphate fractions was examined separately, and one example of this experiment is presented in Table V. Above 90 % of ^{32}P incorporated in the RNA fraction obtained by the method of SCHMIDT-TANNHAUSER is in fact incorporated into the polyphosphate fraction. It was also shown that during 2 h 0.3 % of total phosphate in RNA and 3 % of total phosphate in polyphosphate were labeled with ^{32}P.

The stimulating effect of the factor is predominant on the incorporation of ^{32}P into the polyphosphate fraction.

DISCUSSION

The purified factor which stimulates amylase formation by *B. subtilis* shows the following chemical properties. 1. The active purified factor is reactive only with ninhydrin, and the color developed on paper is red. The rate of reaction with ninhydrin in solution is very slow. 2. It is strongly basic and combines with inorganic phosphate and flavianic acid. 3. On paper chromatography it runs fast in basic solvents and very slowly in acidic solvents. These chemical observations are the characteristic features of alkyldiamines and polyalkylamines. The biological activity of the factor is destroyed by acid and alkali. On the other hand biological alkyldiamines and polyalkylamines, such as 1,3-propandiamine, putrescine, cadaverine, spermidine, and spermine are stable in both acid and alkali. It is assumed, therefore, that the active principle from *B. subtilis* is a derivative of an alkyldiamine or polyalkylamine.

Because the amount of the factor which we have purified so far from *B. subtilis* cells is very small, it is difficult to determine the chemical structure of the factor.

It is well known that alkyldiamines and polyalkylamines exist in bacterial cells[12, 16, 17] and bacteriophage[18].

FRASSA showed the protective effect of spermidine on the infectious factor of phage which is essential in the phage infection of protoplasts[19]. ROSANSKY reported the inhibition of the growth of various bacteria by spermine[3]. Recently MAGER reported the protective effect of spermine on bacterial protoplasts in making them more resistant to osmotic shock[20].

On the mechanism of the biological functions of these amines, however, nothing is known. In this respect, the facts that amine-like material from *B. subtilis* and some natural amines stimulate the exoenzyme formation and metabolism of phosphate compounds of the resting cell of *B. subtilis*, are very interesting.

There are some reports of factors which stimulate protein synthesis and nucleic acid synthesis simultaneously.

GALE AND FOLKES reported that glycine incorporation factor purified from *Staphylococcus aureus* stimulated both the incorporation of glycine into protein and the incorporation of adenine into RNA in the disrupted cells of the same strain[21].

STRAUB showed that arginine is necessary for both amylase formation and the turnover of RNA by a soluble system from pancreas[2]. The effect of the factor from *B. subtilis* is most obvious on the polyphosphate metabolism.

Further there should be some relationship between the metabolism of polyphosphate, the turn-over of RNA and the synthesis of exoenzymes. Further studies on the determination of the chemical structure of the factor and the mechanism of its functions are now in progress.

ACKNOWLEDGEMENTS

The authors wish to express their gratitued to Professor S. AKABORI for his encouragement and stimulating discussion during the course of this study.

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ENZYME SYSTEMS IN THE MYCOBACTERIA

IX. THE REDUCTIVE ACETYLATION OF LIPOIC ACID*

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SUMMARY

The pyruvic dehydrogenase complex of the H37Ra strain of *Mycobacterium tuberculosis* catalyzes the reductive acetylation of lipoic acid. The reaction is inhibited by N-ethylmaleimide and not by arsenite. CoA does not participate in this reaction. A scheme is presented which attempts to unify these and other findings on the mechanism of the oxidative decarboxylation of pyruvate.

If the oxidative decarboxylation of [2-^{14}C]pyruvate is carried out in the absence of external oxidizing agents but in the presence of free acetaldehyde, the external acetaldehyde becomes labeled. This indicates that enzyme-bound acetaldehyde (or a compound which rearranges to acetaldehyde when it dissociates from the enzyme) is an intermediate in the oxidative decarboxylation of pyruvate.

INTRODUCTION

The participation of $lipS_2$ in the oxidative decarboxylation of pyruvate was demonstrated some years ago[3], however, its function was little understood. After the demonstration that "active acetyl" is the thiolester of acetate and CoA (see ref. 4) it was suggested by GUNSALUS[5] and by REED AND DEBUSK[6] that $lip(SH)_2$ serves in an analogous fashion. It was proposed that $lipS_2$ has the dual function of electron acceptor and acetyl acceptor. The product of this reaction, S-acetyl-lip(SH) could not be isolated. REED *et al.*[7] have investigated the role of both free and bound $lipS_2$ in the several reactions involved in pyruvate oxidation (see ref. 8 and 9 for recent reviews of this area of investigation).

We recently described the purification and properties of a soluble pyruvic dehydrogenase complex isolated from the H37Ra strain of *Mycobacterium tuberculosis*[10, 11]. This enzyme complex carries out the reductive acetylation of $lipS_2$ by a mechanism similar to that first proposed by GUNSALUS[5] and by REED AND DEBUSK[8]. Enzyme-bound "acetaldehyde" is an intermediate in this reaction[5, 12]. The reaction

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Abbreviations: $lipS_2$ and $lip(SH)_2$, the oxidized and reduced forms of α-lipoic acid (1,2-dithiolane-3-valeric acid); S-acetyl-lip(SH), 6-S-acetyl-8-mercapto octanoic acid; Tris, tris(hydroxymethyl)amino methane; FAD, flavin adenine dinucleotide; DPN, diphosphopyridine nucleotide.

* For paper 8 in this series see[1]. A preliminary report of these findings has been published[2].

does not appear to involve CoA and, accordingly the mechanism differs from that suggested by SANADI *et al*[13] as applicable to the pyruvic and α-ketoglutaric dehydrogenases of mammalian muscle. While both the animal and the mycobacterial pyruvic dehydrogenases catalyze the oxidative decarboxylation of pyruvate and the formation of S-acetyl-CoA, there appear to be differences in the mechanisms of the reactions catalyzed by the two respective enzymes.

MATERIALS AND METHODS

The sources and preparation of enzymes and substrates, except as mentioned below, and analytical procedures are the same as previously described[10,11]. [2-^{14}C]pyruvate was obtained from Volk Radiochemical, Chicago ;(+)-α-lipS$_2$ was the gift of Dr. D. S. ACKER, duPont de Nemours and Co., and was also obtained by the resolution of commercial (±)-α-lipS$_2$ (see ref. 14). (±)-α-Lipoamide was the gift of Dr. D. R. SANADI and of Dr. A. F. WAGNER, Merck, Sharp and Dohme Research Laboratories.

The assay system for reductive acetylation contains, in a total volume of 0.25 ml, 50 μmoles of Tris buffer of pH 7.5, 4 μmoles of Li-pyruvate, 6 μmoles of (±)-α-lipS$_2$, 0.10 μmole of cocarboxylase, 2 μmoles of MgCl$_2$ and 0.2–2.0 mg of pyruvic dehydrogenase. The tubes are flushed with nitrogen, capped with a rubber stopper and incubated for 30 min at 30°. The tubes are opened and 0.25 ml of neutral, 2.5 *M* hydroxylamine is added. Incubation is continued for an additional 10 min at 22°. The reaction is stopped and the hydroxamic acid color is developed by the addition to the tube of 0.25 ml of 3 *N* HCl and 0.25 ml of 5 % FeCl$_3$. The slightly turbid mixture is thoroughly extracted with 0.5 ml of benzene. The resulting emulsion is clarified by centrifugation (20,000 × *g* for 5 min) and the absorbancy of the aqueous phase at 540 mμ is measured in the Beckman DU spectrophotometer. Under these conditions 1.0 μmole of acethydroxamic acid has a molar absorbancy index of $0.72 \cdot 10^3$ cm^2/mole. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1.0 μmole of acethydroxamic acid under the conditions of the above assay (30 min, 30°, pH 7.5). Specific activity is expressed as units per mg of protein.

RESULTS

Characteristics of the assay system

The dependence of the rate of reductive acetylation on the concentration of substrates and enzyme and on pH and time is shown in Figs. 1 and 2. The highest specific activity routinely obtained ranges from 2 to 4. Although lipS$_2$ inhibits lipoic transacetylase[11] it does not inhibit the reductive acetylation system.

Formation of S-acetyl-lip(SH) from pyruvate

In an experiment, set up on twice the scale of the assay system, 0.76 μmole of an hydroxamic acid and 1.17 μmoles of –SH appeared. A sample of the reaction mixture, before addition of NH$_2$OH, was acidified with HCl and extracted three times with cold benzene. The pooled benzene extracts were evaporated to dryness and the residue was dissolved in methanol. The absorption spectrum of the product, corrected for the blank absorption (determined in control runs without enzyme or pyruvate) is shown in Fig. 3. About 0.51 μmole of S-acetyl-lip(SH) was recovered.

The discrepancy between the amounts of hydroxamic acid and free –SH groups formed is due to the presence of an S-acetyl-lip(SH) deacylase[11].

In a similar experiment, 20 volumes of 95 % ethanol were added to the tube after the reaction with NH_2OH was complete. The precipitate thus formed was removed by centrifugation. The alcohol phase was evaporated to dryness *in vacuo*. The residue was extracted several times with absolute ethanol and the combined ethanol extracts were concentrated *in vacuo*. Acethydroxamic acid was identified in this concentrate by ascending paper chromatography in two solvent systems (*n*-butanol–acetic acid–H_2O, (4:1:5, v/v) and *n*-amyl alcohol–acetic acid–H_2O, (4:1:5, v/v)[15]. The R_F's of the hydroxamate spots obtained by spraying the dried chromatograms with an acidified alcoholic solution of $FeCl_3$ (see ref. 16) were identical with those of known acethydroxamic acid.

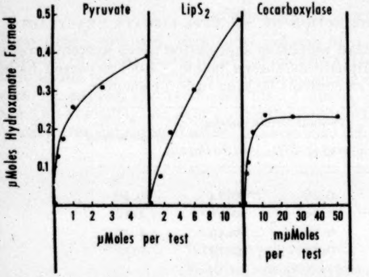

Fig. 1. Dependence of the rate of reductive acetylation on the concentrations of pyruvate, $lipS_2$ and cocarboxylase.

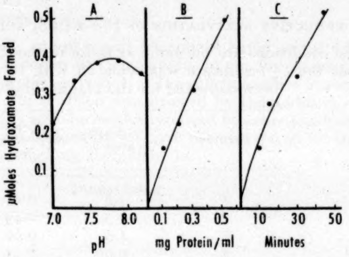

Fig. 2. Effect of pH, protein concentration and time on the reductive acetylation of $lipS_2$.

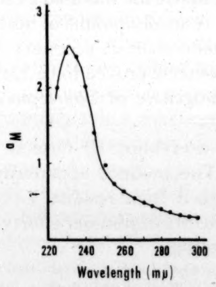

Fig. 3. Absorption spectrum of the product of the reductive acetylation of $lipS_2$.

(+)-α-*lipoate as substrate of the pyruvic dehydrogenase*

Attempts to show complete conversion of (+)-α-$lipS_2$ to S-acetyl-lip(SH) were unsuccessful due to the presence of S-acetyl-lipoate deacylase (eqn. 1).

$$\text{S-acetyl-lip(SH)} + H_2O \longrightarrow \text{acetate} + \text{lip(SH)}_2 \qquad (1)$$

A maximum of 70 % of the added (+)-α-$lipS_2$ was converted to the acetyl derivative.

That the acetylation was actually greater than this, is shown by the experiments described in Table I. (—)-α-lipS_2 was active in the reductive actylation system only to the extent that it was contaminated with (+)-α-lipS_2.

TABLE I

REDUCTIVE ACETYLATION OF (+)-α-lipS_2 AND THE ACTION OF S-ACETYL-LIPOATE DEACYLASE

The conditions are the same as those in the standard reductive acetylation assay system except that the (±)-α-lipoate was replaced with (+)-α-lipoate as shown below. Values reported have been corrected for the blanks observed in controls lacking lipS_2 or enzyme.

Expt. No.	*(+)-α-lipS_2 (μmoles)*	*Pyruvic dehydrogenase* (mg)*	*Products formed (μmoles)*		*Lipoate utilized*
			S-acetyl-lip(SH)	*Lip(SH)$_2$*	
1	1.5	0.23	0.26	0.14	0.40
2	1.5	0.45	0.66	0.46	1.12
3	1.5	0.60	0.76	0.49	1.25
4	0.90	0.45	0.34	0.23	0.57

* Specific activity 1.0.

Experiments in which (±)-α-lipoamide was used as substrate for reductive acetylation gave questionable results. A small amount of acetylated product was formed. The water insolubility of the amide made it necessary to use an ethanolic solution of the compound. Unfortunately ethanol proved to be highly inhibitory to the system. By contrast the pyruvic dehydrogenase of *Streptococcus faecalis* is unaffected by ethanol[9].

Attempts to isolate pure S-acetyl-lip(SH) formed by reductive acetylation of (+)-α-lipS_2 were unsuccessful. The product apparently polymerized and became insoluble during attempts to free it from residual (+)-α-lipS_2 which is highly inhibitory to the transacetylase. This frustrated our efforts to use pure S-acetyl-lip(SH) as an acetyl donor in coupled reactions.

Effect of potential inhibitors on the reductive acetylation reaction

Reductive acetylation of lipoate is not inhibited by $4 \cdot 10^{-2}$ M ethylenediaminetetraacetate or $1 \cdot 10^{-2}$ M arsenite. N-ethylmaleimide at $6 \cdot 10^{-3}$ M inhibits about 80 %.

Formation of acetoin

The pyruvic dehydrogenase of H37Ra can condense acetaldehyde (externally added) with "active acetaldehyde" (enzyme-bound) in the absence of lipoate. Under these conditions acetoin is formed. The requirements for acetoin formation are shown in Table II.

Demonstration of (E-acetaldehyde) as an intermediate in pyruvate oxidation

In these experiments the decarboxylation of pyruvate was carried out in the absence of an oxidizing agent (lipS_2) but in the presence of free acetyladehyde. [2-^{14}C]pyruvate was the substrate, [1-^{14}C]acetaldehyde was expected as the product. The reaction mixture was flushed with N_2 and incubated for 15 min at 38°. The reaction was stopped by the addition of $HClO_4$ and the acidified mixtures were held at 0° for 10 min before neutralization with KOH and centrifugation. A sample of the neutral supernatant solution was analyzed for the presence of acetaldehyde by the diffusion method[10]. The sample (0.4 ml) was mixed with 50 μmoles of pH 7.0 phosphate buffer in the diffusion bulb and frozen in acetone–ice. The trapping tube contained 0.20 ml of 1.0 % dinitrophenylhydrazine. Diffusion was carried out for 30 min at 38° under reduced pressure. After the diffusion assemblies were opened, 0.5 ml of 1.0 % dinitrophenylhydrazine was added to each diffusion bulb. The hydrazones were formed during a 10 min incubation at room temperature. The hydrazones in the diffusion bulbs and trapping tubes were extracted with ethyl acetate. The dinitrophenylhydrazones were separated and identified by ascending paper chromatography. Three solvent systems were used: (a) *n*-butanol–ethanol–NH_4OH–H_2O (140:20:1.5:38), (b) *tert.*-amyl alcohol–ethanol–H_2O (100:20:80) and (c) ethanol–petroleum ether (80:20). The following standards, as the dinitrophenylhydrazones, were always chromatographed along with the experimental and control tube samples: [2-^{14}C]pyruvate, diacetyl, acetaldehyde and acetoin as well as dinitrophenylhydrazine itself. Samples and standards were applied to the origin as one inch streaks. After development of the chromatogram the papers were dried in air. The chromatogram developed by each streak was removed by cutting the paper into vertical strips; each strip was cut in half. The yellow spots on the first half were intensified and the characteristic colors of the dinitrophenylhydrazones were brought out by spraying the dried strip with a solution of 1 % KOH in 95 % ethanol. The second half of the streak was monitored for ^{14}C-containing areas by using a recording strip counter*.

TABLE II

FORMATION OF ACETOIN FROM PYRUVATE

Each tube contained, in a total volume of 0.70 ml, 40 μmoles of phosphate buffer of pH 6.8, 5 μmoles of $MgCl_2$, 0.005 μmole of cocarboxylase, 4 μmoles of Li-pyruvate, 40 μmoles of acetaldehyde and 0.46 mg of pyruvic dehydrogenase of specific activity 1.7. The solutions were flushed with N_2, capped and incubated for 15 min at 38°. The reaction was stopped by the addition of 0.020 ml of $HClO_4$; the acid was neutralized with KOH and the residue removed by centrifugation. Acetoin was determined after conversion to diacetyl[16]. Values shown have been corrected for the blank obtained in a control run lacking acetaldehyde.

System	*Diacetyl formed (μg)*
Complete	12.3
Without pyruvate	5.1
Without cocarboxylase	1.3
Without $MgCl_2$	4.1
Without pyruvic dehydrogenase	0.69

Aliquot samples of the diffusion bulb and tube ethyl acetate solutions, from above, were plated on aluminum planchets and total ^{14}C contents were measured in a flow-gas counter. Results of a typical experiment are shown in Table III and Fig. 4. Control experiments showed that while negligible amounts of pyruvate diffuse from bulb to collecting tube under these conditions, essentially all the acetaldehyde diffuses.

These experiments can be summarized as follows. A strongly-labeled non-diffusible compound is formed non-enzymically. The R_F of its dinitrophenylhydrazone in all systems is identical with that of a non-radioactive dinitrophenylhydrazone which

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* I wish to thank Dr. J. W. PORTER for making the radioisotope-detecting equipment available to me for these experiments.

TABLE III

EXCHANGE OF POOL ACETALDEHYDE WITH ENZYME-BOUND ACETALDEHYDE

Each tube contained, in a total volume of 0.60 ml; 30 μmoles of phosphate buffer of pH 6.9, 3 μmoles of $MgCl_2$, 0.003 μmole of cocarboxylase, 4 μmoles of Li-pyruvate, 2.0 μmoles of Na-[2-^{14}C] pyruvate (3.17 μC/μmole), 40 μmoles of acetaldehyde and 0.31 mg of pyruvic dehydrogenase of specific activity of 1.7.

System	*^{14}C recovered*		
	Non-diffusible (counts/min)	*Diffusible*	
		counts/min	*Percent*
Complete	$9.06 \cdot 10^5$	$24.8 \cdot 10^3$	2.7
Without enzyme	$8.76 \cdot 10^5$	$1.25 \cdot 10^3$	0.14
Without cocarboxylase	$9.60 \cdot 10^5$	$0.29 \cdot 10^3$	0.03
Without $MgCl_2$	$7.56 \cdot 10^5$	$9.51 \cdot 10^3$	1.3

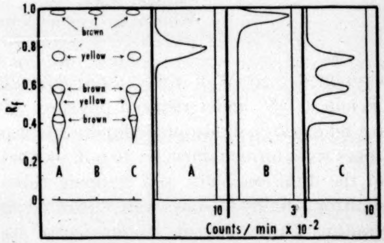


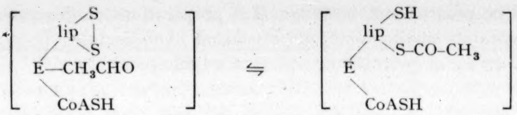
Fig. 4. Detection of [^{14}C]acetaldehyde by paper chromatography (left) and strip counting (right). Experiments as described in the text. A. 2,4-dinitrophenylhydrazones recovered from the nondiffusible residue of a control run (without enzyme). The single radioactive peak corresponds to that of [2-^{14}C]pyruvate. B. 2,4-dinitrophenylhydrazone of a diffusible compound recovered from the complete reaction system. C. 2,4-dinitrophenylhydrazones of the nondiffusible ^{14}C-containing compounds formed in the complete reaction system.

contaminates the dinitrophenylhydrazone of [2-^{14}C]pyruvate. Its R_F is less than that of the [2-^{14}C]pyruvate derivative. In the presence of the complete enzymic system a diffusible radioactive compound is formed which reacts with 2,4-dinitrophenylhydrazine. The R_F of its hydrazone is identical with that of the dinitrophenylhydrazone of acetaldehyde in three solvent systems. When the same experiment is carried out in absence of either enzyme or cocarboxylase this diffusible substance is not found. In the presence of carrier acetaldehyde the dinitrophenylhydrazone can be recrystallized (ethanol–water) to constant specific activity. Three recrystallizations are usually sufficient.

Despite the fact that free acetaldehyde exchanges with enzyme-bound "acetaldehyde" it was not possible to use acetaldehyde as an acetyl donor for lipoate.

Role of CoA in the reductive acetylation reaction

SANADI *et al.*[13] suggested that S-acetylCoA is an obligatory intermediate in the reductive acetylation of lipS_2. They proposed that the acetyl group of the initial enzyme-bound S-acetyl-lip(SH) is transferred to enzyme-bound CoA. The S-acetyl-CoA is then the substrate for the lipoic transacetylase which transfers the acetyl group to lip$(SH)_2$. This sequence is shown below. A similar sequence showing a requirement for added CoA was presented by REED *et al.*[7].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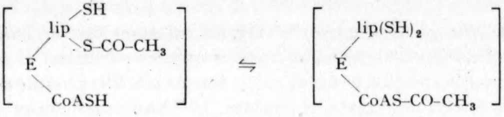
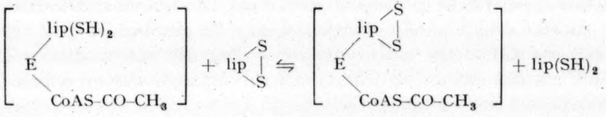

(3)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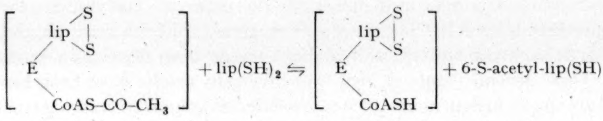

(5)

Our results do not support this proposal. (a) Treatment of the pyruvic dehydrogenase with Dowex-1 to remove CoA (see ref. 18) had no effect on the reductive acetylation reaction. (b) If acetyl CoA is formed in this reaction we should be able to transfer its acetyl group to phosphate by the addition of phosphotransacetylase and phosphate.

The acetyl group of acetyl P is readily hydrolyzed under conditions of temperature and pH which do not affect the acetyl group of S-acetyl-lip(SH). When this experiment, shown in Table IV, was carried out, all the hydroxamate formed was heat and acid-stable. In the absence of $lipS_2$ no hydroxamate was formed. Addition of free excess CoASH did not change this result. It would appear that S-acetyl-CoA is not an intermediate in the reductive acetylation reaction.

TABLE IV

EFFECT OF CoA ON THE REDUCTIVE ACETYLATION OF $lipS_2$

The standard reaction mixture with 0.31 mg of pyruvic dehydrogenase of specific activity 2.5, was used. Additions to the mixture are shown below.

Expt. No.	*Additions*	*Heat treatment*	*µmoles hydroxamate formed*
1	None	None	0.82
2	Phosphotransacetylase* + 5 µmoles Pi	None	0.65
3	None	100° for 3 min	0.64
4	Phosphotransacetylase + 5 µmoles Pi	100° for 3 min	0.69
5	0.05 µmole CoASH	None	0.72
6	Phosphotransacetylase + 5 µmoles Pi + 0.05 µmole CoASH	None	0.80
7	0.05 µmole CoASH	100° for 3 min	0.70
8	Phosphotransacetylase + 5 µmoles Pi + 0.05 µmole CoASH	100° for 3 min	0.64

* 0.89 µg of specific activity 8.4 (see ref. 18).

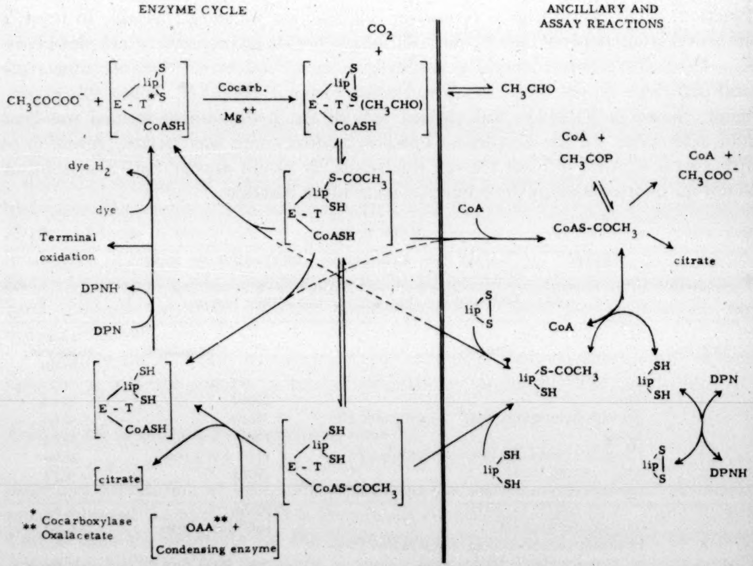
It should be pointed out, however, that preparations of this pyruvic dehydrogenase may contain a small amount of bound CoA. A typical dehydrogenase preparation contained 20 μg of pantothenic acid/g of soluble protein*.

DISCUSSION

The pyruvic dehydrogenase complex of H37Ra catalyzes the fundamental reaction characteristic of all pyruvic dehydrogenase complexes regardless of source, namely the oxidative decarboxylation of pyruvate to acetyl CoA. The product may be isolated as acetyl CoA, acetyl phosphate or acetate; the basic mechanism of the overall reaction appears to be the same whichever product is formed. The differences between the various dehydrogenase complexes may be ascribed, in part, to the isolation procedures and to the assay procedures. Thus the intermediation of $lipS_2$ in the overall reaction cannot be demonstrated if the assay system is based on reduction of indophenol or evolution of CO_2.

Previous attempts to demonstrate the reductive acetylation reaction have led to questionable results. SANADI *et al.*[13] suggested that this reaction, recently reported by GUNSALUS AND SMITH[9], is an artifact arising from reactions 2–5, above.

These several points of view and divergent results have been brought together and are shown in Scheme 1 which is presented as an overall unifying reaction sequence. Scheme 1 is obviously drawn from earlier suggestions[9, 13, 20] and is the result of many investigations, some of which have already been mentioned. The present investigation


ENZYME CYCLE
ANCILLARY AND ASSAY REACTIONS
CO2 +
CH3COCOO⁻ +
E - T* lip S S CoASH
Cocarb.
Mg⁺⁺
E - T - (CH3CHO) lip S S CoASH
CH3CHO
E - T lip S-COCH3 SH CoASH
CoA +
CH3COP
CoA +
CH3COO⁻
CoA
CoAS-COCH3
citrate
dye H2
dye
Terminal oxidation
DPNH
DPN
lip S S
CoA
lip S-COCH3 SH
lip SH SH
DPN
DPNH
E - T lip SH SH CoASH
citrate
E - T lip SH SH CoAS-COCH3
lip SH SH
lip S S
* Cocarboxylase
** Oxalacetate
OAA** +
Condensing enzyme

Scheme 1

---

* I am grateful to the Wisconsin Alumni Research Foundation for this analysis.

provides information relative to the S → S acyl transfer step. (The reaction in which the acetyl group of enzyme-bound S-acetyl-lip(SH) is transferred to external lipS_2.)

Reed *et al.*[7] showed that during the course of the oxidative decarboxylation of pyruvate by the apopyruvic dehydrogenase of *Escherichia coli* (a) there is no exchange between enzyme-bound lipS_2 and free $\text{lip(S}_2)$, lip(SH)_2, or oxidized and reduced lipoamides; (b) CoA is required for the overall reaction leading to the formation of acetyl phosphate; (c) interaction is possible between protein-bound and free $\text{lip(S}_2)$. In the absence of free CoA a very small (2 %) amount of hydroxylamine-reacting product was formed. This was assumed to be S-acetyl-lip(SH). These observations, taken with the results of Sanger[21], Ryle and Sanger[22] and Thomas and Reed[23], suggest a possible alternative to the mechanism suggested by Reed *et al.*[7] and Sanadi *et al.*[13] for the oxidative decarboxylation of pyruvate.

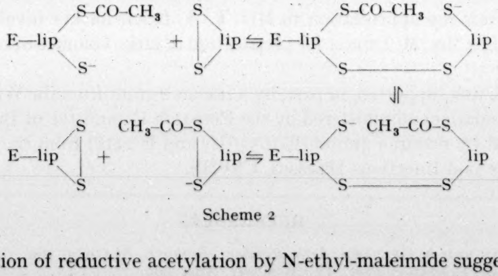
During an investigation by Sanger on the structure of insulin[21] it was shown that disulfide exchange reactions occur. This was confirmed in model systems[21, 22]. Of particular interest was the finding that disulfide exchange reactions occur only slowly at neutral pH and that the reaction is strongly stimulated by the addition of thiols. The catalytic effect of thiols is reversibly inhibited by N-ethyl-maleimide. Ryle and Sanger suggested the following mechanism for the thiol catalysis of disulfide exchange.

$$R^1SSR^1 + R^2S^- \rightleftharpoons R^1SSR^2 + R^1S^- \qquad (6)$$

$$R^2S^- + R^1SSR^2 \rightleftharpoons R^2SSR^2 + R^1S^- \qquad (7)$$

Thomas and Reed[23] investigated the mechanism of the depolymerization of polylipS_2. Depolymerization at room temperature is dependent on the presence of OH^-. At pH 12 depolymerization is markedly stimulated by the presence of catalytic amounts of lip(SH)_2. *p*-Chloromercuribenzoate completely inhibits depolymerization; lip(SH)_2 reverses the inhibition. A mechanism similar to that proposed by Ryle and Sanger was suggested by Thomas and Reed to account for the catalytic effect of mercaptide ions on the depolymerization of polylipS_2.

This suggests that the pyruvic dehydrogenase complex of H37Ra may carry out a disulfide exchange reaction between bound S-acetyl-lip(SH) and free lipS_2. S-acetyl-lip(SH) can contribute the mercaptide ion to the reaction (Scheme 2). An S → S acetyl transfer is not uncommon in biological systems, *e.g.*, the transfers catalyzed by CoA-lip(SH)_2 transacetylases[11, 13, 24] and by the general CoA-thiol transacetylase of Brady and Stadtman[25].



Scheme 2

The inhibition of reductive acetylation by N-ethyl-maleimide suggests the intermediation of a monothiol whereas the lack of inhibition by arsenite would preclude the participation of a dithiol in the reaction. The effect of arsenite in suppressing the production of acetyl CoA by this same pyruvic dehydrogenase of H37Ra can be explained in terms of the binding of enzyme-bound lip$(SH)_2$ formed after transfer of the acetyl group to exogenous CoA. We have previously shown[10] that there is an absolute requirement for CoA for citrate formation by the pyruvic dehydrogenase of H37Ra supplemented with oxalacetate and condensing enzyme. This reaction is strongly inhibited by arsenite.

The picture of pyruvic oxidative decarboxylation by the enzyme of H37Ra which emerges from these studies differs only in detail from the schemes suggested earlier. It may, indeed, differ only in the binding strength between the several enzymes of the complex. Scheme 1 supplies a possible general explanation for observed results by segregating the enzyme-bound transfer and oxidation–reduction reactions from the artificial *in vitro* reactions. By extension of this scheme the results of most investigations can be explained in terms of siphoning off of various intermediates in the decarboxylation cycle into artificial assay systems.

The recent report of GRUBER AND WASSENAAR[26] on the inhibition of yeast carboxylase by acetaldehyde may provide an explanation for the inability of acetaldehyde to serve as an acetyl donor for lipoate in the pyruvic dehydrogenase system. SEARLS AND SANADI[27] and MASSEY[28] have independently shown that the FAD of pig heart ketoglutaric dehydrogenase is associated with the lipoyl dehydrogenase portion of the ketoglutaric dehydrogenase complex. MASSEY[29] has shown the identity of this lipoyl dehydrogenase with the STRAUB[30] diaphorase. MASSEY[28] has suggested that the ketoglutaric dehydrogenase goes through a cyclical reaction sequence similar to that proposed above using the pyruvic dehydrogenase complex as the model. Succinyl is thus generated and simultaneously transferred to E-lipS_2 and, in turn, is transferred from the enzyme-bound succinyl lipoate to CoA yielding succinyl CoA. The only difference of consequence between the cyclical mechanism suggested by MASSEY[28] and that suggested here is that MASSEY proposes that DPN is the physiological oxidant of the reduced flavin of lipoyl dehydrogenase. It appears reasonable to assume, however, that once the FAD of lipoyl dehydrogenase is reduced it is reoxidized *via* the cytochrome sequence rather than *via* DPN, FAD and then the cytochrome sequence[31].

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QUANTITATIVE DETERMINATION OF N-TERMINAL AMINO ACIDS IN SOME SERUM PROTEINS

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SUMMARY

A method for quantitative determination of N-terminal amino acids of proteins is described. Results obtained from different serum protein fractions are given and compared with published data.

INTRODUCTION

During a chemical study of different serum proteins it was found desirable to have a reliable method for the quantitative determination of their N-terminal amino acids. Hitherto, the DNP-method of SANGER[1] has been generally used[2-7]. Although excellent results have been obtained by SANGER's technique, it has the disadvantage of giving low recoveries because of losses of the DNP-amino acids during acid hydrolysis. EDMAN's thiohydantoin method[8], however, has proved satisfactory for the sequence determination of peptides, but this method has been infrequently used with higher molecular weight proteins, because of the reported low recoveries of N-terminal amino acids. The losses depend partly on destruction of the PTH's during the cyclization step, and also on difficulties with the determination of the amino acids released.

This paper reports an adaptation of the EDMAN technique for various serum proteins and has so far given quantitative recoveries of the N-terminal amino acids. The final quantitative determination of the PTH's has been performed using the paper chromatographic technique recently described by SJÖQUIST[9].

MATERIAL AND METHODS

Human serum albumin was prepared according to a modified COHN method 10. It moved as a homogeneous peak in free electrophoresis. Nitrogen content: 15.0 %.

Human γ-globulin (COHN fraction II) was prepared according to the modified procedure of DEUTSCH *et al.*[10]. It contained about 2 % each of albumin and β-globulin. Nitrogen content: 15.5 %. The γ-globulin was used without further purification.

Porcine γ-globulin (COHN fraction II) was a commercially available preparation

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Abbreviations: DNP, dinitrophenyl-; PTH, phenyl thiohydantoin-; PTC, phenyl thiocarbamyl-.

from Pentex Inc., Kankatee, Ill. (U.S.A.), which was homogeneous by paper electrophoresis. Nitrogen content: 15.5 %.

Bovine prothrombin was prepared essentially according to the method of SEEGERS[11] and further purified by chromatography on Amberlite XE 64 according to the method of MILLER[12]. Nitrogen content: 13.3 %.

Human β_1-iron binding globulin (transferrin) was prepared by a modification of COHN fraction IV:7 and further purified by chromatography on DEAE-cellulose[13]. The preparation was homogeneous by starch gel electrophoresis[13]. Nitrogen content: 14.4 %.

Methylethyl ketone (Merck *p.a.*) was refluxed with $KMnO_4$ and distilled.

Pyridine (Merck *p.a.*) was refluxed over phthalic acid anhydride and distilled.

Other reagents used in the present investigation were purified as described in a previous paper[14].

Nitrogen determinations were performed using a micro Kjeldahl method.

Extinction readings were performed in a Beckman DU spectrophotometer.

Assay of N-terminal amino acids

Thick walled Pyrex tubes (length 13 cm, outer diameter 2.5 cm, provided with a male joint B29) designed to fit a lyophilizer were used at all times. Washings and other mixing operations were achieved with the aid of a Teflon coated magnet which was left in the tube through the whole experiment.

The protein sample (5–20 mg depending on molecular weight and computed amount of N-terminal amino acids) was dissolved in 1 ml saline and 2 ml of a solution of pyridine–triethylamine–phenyl isothiocyanate (100:3:1) was added. After mixing, the tube was placed in a water bath at 40° for 1.5 h. The solution, now containing the protein as PTC derivative, was thoroughly washed five times with 5-ml aliquots of benzene–ethylene chloride (3:1) previously saturated with 0.1 *N* NaOH. After centrifugation the benzene–ethylene chloride phase was discarded. The tube was subsequently placed at 40° in a gentle stream of N_2 to remove the last traces of organic solvent. In order to split off the N-terminals from the PTC-protein, 1 ml water and 2 ml hydrogen chloride saturated acetic acid were added with thorough mixing and the tube was kept at 40° for 2 h. Subsequently, the sample was lyophilized. Lyophilization was interrupted before the sample was completely dry, thereby preventing the protein's adherence to the glass walls. The residue was suspended in 2 ml water previously saturated with ethyl acetate–methylethyl ketone (2:1). The PTH's were extracted from the aqueous phase by four 2-ml extractions with ethyl acetate–methylethyl ketone (2:1) saturated with water. The extracts were combined and taken to dryness at 40° in a stream of N_2. The residue was dissolved in 0.1 ml 90 % acetic acid and suitable aliquots (10–40 μl) were used for paper chromatography according to the method recently described[9]. The PTH's were then eluted from the chromatograms at 40° with 2 ml 95 % ethanol for 1 h. The amounts of N-terminal amino acids present were calculated from the u.v. absorption at 269 (or 320) mμ. In these calculations the coefficients for recovery of each amino acid were used which were specified in a preceding paper[9].

No estimations of arginine or histidine were performed, as the PTH derivatives of these amino acids are water soluble and thus remain in the aqueous phase during the extraction.

RESULTS

Several attempts were made to find out the optimal conditions for the PTC-coupling and cyclization as well as washing and extractions. As test protein in these experiments human γ-globulin generally was used as it is known to have predominantly N-terminal aspartic acid and glutamic acid. Fig. 1 is a representative experiment, and shows the final recoveries of N-terminal aspartic acid and glutamic acid after different cyclization times. It is evident that a cyclization time of 2 h is sufficient. The values also indicate that prolonged cyclization does not destroy the PTH's to any appreciable extent. However, cyclization, lyophilization and extraction of the PTH derivatives should be performed during the same day. In some instances, considerable losses of the PTH derivatives occurred when the sample after lyophilization was kept at —20° overnight. On the other hand, after PTC coupling and washing, the sample can be kept at —20° overnight without losses.

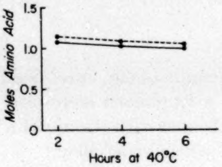

Fig. 1. Recoveries of N-aspartic acid, ———, and N-glutamic acid, – – – –, from human γ-globulin after different cyclization times. The values are expressed as moles amino acid/160,000 g γ-globulin.

Fig. 2 shows a paper chromatogram of the PTH amino acids obtained from human γ-globulin, as well as PTH derivatives of known amino acids for comparison. Fig. 3 compares the u.v. absorption spectra of the eluted spots with the spectrum of authentic PTH aspartic acid. (The spectra of PTH aspartic acid and PTH glutamic acid are virtually identical.) Undoubtedly, the eluted spots are PTH derivatives of glutamic acid and aspartic acid originating from the N-terminals of the γ-globulin fraction. The identity was also checked by hydrolyzing the PTH's and subsequently identifying the free amino acids by paper chromatography.

The small peak at 320 mμ of the eluted spot (Fig. 3) is due to a small but significant amount of PTH serine migrating with PTH aspartic acid on the chromatogram. Although these two PTH derivatives have the same R_F on the chromatogram, each can be individually determined because of the extra absorption of PTH serine at 320 mμ. Details for calculation of the serine content are given in a preceding paper[9].

The reproducibility of this method is shown in Table I, where eight different analyses on the same γ-globulin were performed.

Table II shows the N-terminal amino acids of the different serum proteins studied. No other N-terminal amino acids were detected than those indicated. The molar content of each amino acid was calculated from the published mean molecular weight of each protein fraction. As expected, human albumin had 1 mole aspartic acid/mole protein. Human γ-globulin contained 1 mole each of aspartic acid and glutamic acid and consistently a small amount of serine. Porcine γ-globulin, however, had 1 mole alanine and 2 moles of glutamic acid/mole of protein. Human transferrin showed only valine as an N-terminal amino acid in a ratio of 1 mole/mole protein. Bovine prothrombin contained 1 mole of alanine/mole protein. These figures are rounded off to the nearest stoichiometric value although the protein fractions used were not completely homogeneous.

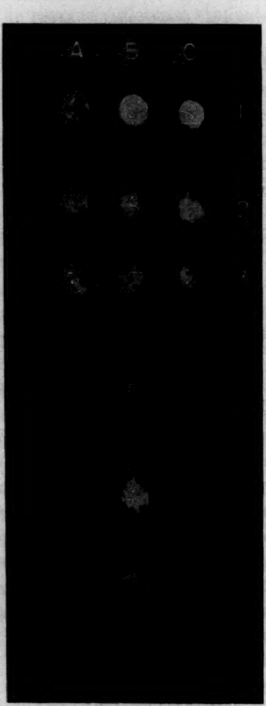

Fig. 2. Paper chromatogram of N-terminal amino acids from human γ-globulin (A and C). B denotes test strip. The figures indicate the following: 1, application point; 2, PTH-aspartic acid and PTH-serine; 3, PTH-glutamic acid.

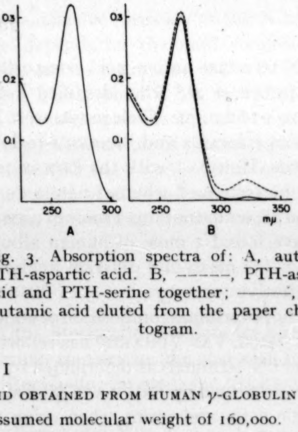

Fig. 3. Absorption spectra of: A, authentic PTH-aspartic acid. B, – – – – –, PTH-aspartic acid and PTH-serine together; ———, PTH-glutamic acid eluted from the paper chromatogram.

TABLE I

MOLES N-ASPARTIC ACID AND N-GLUTAMIC ACID OBTAINED FROM HUMAN γ-GLOBULIN

The values have been calculated from an assumed molecular weight of 160,000.

Expt. No.	*γ-globulin (mg)*	*Moles N-terminal amino acids per 160,000 g protein*	
		Aspartic acid	*Glutamic acid*
1	16.95	1.15	1.22
2	16.00	1.11	1.12
3	28.25	1.05	1.20
4	21.01	1.13	1.20
5	21.01	1.07	1.15
6	21.01	1.15	1.20
7	21.01	1.18	1.18
8	21.01	1.10	1.14
Mean		1.12	1.18

TABLE II

N-TERMINAL AMINO ACIDS OF DIFFERENT SERUM PROTEINS

The values have been expressed as moles amino acid/mole protein.

Protein	*Assumed molecular wt.*	*Number of determinations*	*Alanine, moles*	*Aspartic acid, moles*	*Glutamic acid, moles*	*Serine, moles*	*Valine, moles*
Albumin (human)	69,000	4	—	1.03	—	—	—
γ-globulin (human)	160,000	8	—	1.12	1.18	0.10	—
γ-globulin (porcine)	160,000	4	1.04	—	1.99	—	—
Prothrombin (bovine)	62,700	2	1.10	—	—	—	—
Transferrin (human)	88,000	2	—	—	—	—	0.98

DISCUSSION

The N-terminal amino acid composition of human albumin has been studied by DESNUELLE *et al.*[2] who identified aspartic acid as the N-terminal amino acid by SANGER's technique. Their results were later confirmed by THOMPSON[3] in a comparative study of EDMAN's and SANGER's technique. THOMPSON found aspartic acid by both methods. However, with the EDMAN technique only 5 % of N-terminal aspartic acid could be recovered whereas SANGER's technique gave 64 % on the assumption that human albumin contains 1 mole of aspartic acid/mole albumin. By the present method we have found 1 mole of human albumin to contain 1.03 moles aspartic acid. This value is the mean of 4 different analyses. These results suggest the method to give a quantitative recovery.

The N-terminal composition of γ-globulin from various species has been thoroughly investigated. VAN VUNAKIS[4] has reported human γ-globulin to contain at least seven different N-terminals as determined by the DNP-method. However, this finding could not be confirmed by SMITH AND MCFADDEN[5]. These authors found only glutamic acid, aspartic acid, and serine when using the same technique. Certain differences were found to exist between subfractions of human γ-globulin. Thus fraction II: 1,2 gave 1.06 moles aspartic acid, 1.82 moles glutamic acid and 0.10 moles serine whereas fraction II: 3 contained 1.01 moles, 1.06 moles and 0.17 moles respectively. The values were based on the recoveries of the DNP-aspartic acid (60 %), DNP-glutamic acid (56 %) and DNP-serine (81 %) and on an assumed molecular weight of γ-globulin of 160,000. Recently LAY AND POLGLASE[7] have determined both the N-terminal and C-terminal amino acids of a human γ-globulin fraction. After correction for losses of the DNP-amino acids during hydrolysis they report one mole each of N-aspartic acid and N-glutamic acid. No N-serine was found.

In our investigation the human γ-globulin revealed 1.12 moles N-aspartic acid, 1.18 moles N-glutamic acid and approx. 0.1 moles N-serine. In this respect our results agree well with the findings of SMITH AND McFADDEN for fraction II:3 and with those of LAY AND POLGLASE (except for the serine content). However, our values are not corrected for losses as we assume the procedure to give quantitative recoveries of the N-terminal amino acids also from human γ-globulin.

The porcine γ-globulin studied has been found to contain 1.04 moles alanine and 1.99 moles glutamic acid. COLACICCO[15] has studied the N-terminal composition of this protein fraction (Pentex Inc.) by the DNP-method. He has found the ratio between N-glutamic acid and N-alanine to be 2:1. After correction for losses of the DNP amino acids by 30 %, the protein contains 2 moles N-glutamic acid and 1 mole alanine. However, he found small amounts of serine, threonine, valine and isoleucine which we cannot verify in our protein preparation.

We cannot compare our finding of 1 mole N-valine/mole transferrin to other published data as the only data available is that of PUTMAN[16] who has reported human transferrin to contain N-valine, but did not give quantitative data.

Assuming a molecular weight of 62,700 for bovine prothrombin we found 1.10 moles alanine/mole protein. This value is higher than the results of MAGNUSSON[17] who found 0.95 moles N-alanine in the same preparation/mole protein. He used a modification of EDMAN's method[18] and corrected for losses during hydrolysis from added known amounts of alanine.

The method presented appears to give quantitative recovery of the N-terminal amino acid from proteins. This presumably depends on the mild conditions for splitting off the N-terminals and on the reliability in the estimation of the PTH amino acids by the paper chromatographic technique. The method is not restricted to the determination of the N-terminal amino acids of a single protein. It could be used as a suitable method for checking the purity during fractionation of protein as well as a simple technique for molecular weight estimate. We have applied this method for determination of all of the N-terminal amino acids of dialyzed whole serum from normal and pathological sera. This has given interesting results which will be published.

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THE ACTIVATION OF CARNOSINASE BY DIVALENT METAL IONS

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SUMMARY

1. A cell and a procedure for the electrodialysis of protein solutions has been described which is suitable for the removal of stabilizing Mn^{2+} ions from solutions of purified carnosinase. The rate of dialysis obtained is considerably higher than the rate of inactivation of the metal-ion free enzyme.

2. It has been shown that Mn^{2+} ions, besides stabilizing the enzyme, also act as activators, performing the role of a coenzyme for the enzymic reaction.

3. Of the divalent metal ions investigated Cd^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} are shown to function as activators for carnosinase. The apparent efficiency of activation is given by the order: $Cd^{2+} > Mn^{2+} \gg Zn^{2+} > Co^{2+}$.

4. The Mn^{2+} activated enzyme is inhibited both by ions functioning as activators, such as Zn^{2+} and Co^{2+}, and by non-activating ions with Be^{2+} and Fe^{2+} as the most effective inhibitors.

5. The time course of the enzymic reaction in the presence of Mn^{2+}, Cd^{2+} and Zn^{2+} is shown to be of different type for each of the ions. The factors influencing the course of the reaction are discussed.

6. The influence of the concentration of free Mn^{2+} and Cd^{2+} ions on carnosinase activity has been studied. The results are, in the case of Cd^{2+}, in accordance with the assumption that one Cd^{2+} ion interacts with each active site. The influence of Mn^{2+} ions on the measured activity is most simply explained, by assuming the necessity for two metal ions per active site. It is suggested that the second Mn^{2+} ion is necessary for stabilizing the enzyme molecule.

7. The metal-ion specificity of carnosinase and its dependence on the properties of the metal ions and the conditions of the assay have been discussed.

INTRODUCTION

Among the enzymes classified as metal-ion activated, the peptidases occupy a central position. Extensive investigations by SMITH and co-workers[1-5], who used mainly partially purified enzyme preparations, established substrate and metal-ion specificity for a large number of peptidases. These investigators found that all the peptidases examined exhibited a very high degree of specificity towards the activating metal ions. Carnosinase[1], prolidase[3], prolinase[4], glycylglycinedipeptidase[5] and leucine aminopeptidase[2] were all shown to be activated by only one or possibly two different

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Abbrevations: EDTA, ethylenediamine tebracetate; THAM, tris(hydroxymethyl)aminomethane.

divalent metal ions. Such a high degree of specificity contrasts markedly with the specificity shown by other groups of metal-ion activated enzymes. Enolase, for example, thoroughly studied by a number of investigators[6,7], is activated to different degrees by Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cd^{2+} and Ni^{2+}. Such a broad spectrum of activation has recently been shown to be characteristic even for metallo-enzymes such as carboxypeptidase[8]. In this case, although the activity is independent of the free metal ion concentration due to extremely strong binding of the metal ion, the Zn^{2+} originally bound to the enzyme can be replaced by Co^{2+}, Cr^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+} without the enzyme losing its catalytic properties. If the narrow metal-ion specificity of peptidases is accepted as definite, extremely selective restrictions must be imposed on the properties of the activating ion by the structure of the enzyme and the mechanism of the enzyme action. On the other hand the picture is complicated by the instability of the enzyme in solution and the observed metal-ion stabilization. For example in the case of carnosinase[1,9] Mn^{2+}, besides activating the enzyme, also functions as a stabilizer. An examination of available data on metal-ion activation of enzymes[10] reveals that in general apparent activation of an enzyme can often be adequately explained by metal-ion stabilization of the enzyme structure against thermal denaturation at the temperature of activity measurements. In this case, even if the role of the metal ion is essential and specific there is no need to postulate the existence of an enzyme–metal-substrate compound; indeed the metal ion need not be located at the active site of the enzyme or partake directly in the process of catalysis.

If the metal ion acts as a stabilizer, possible irreversible and reversible steps leading to inactivation may render the results of the activity measurements very sensitive to the conditions and handling of the enzyme sample prior to the assay. The temperature and duration of steps such as dialysis and incubation with metal ions have a considerable influence on the results. Because of this, the apparent specificity obtained when no special precautions were used to control inactivation may often be misleading. As a part of a research program on the properties and mechanism of the action of metal enzymes being carried out at this institute, it seemed advisable to re-investigate the metal-ion activation of peptidases in a more careful and systematic way, so that it may be possible to differentiate between stabilization and true activation by metal ions. Further results ought to indicate whether there should be postulated a type of mechanism for metal-ion activation essentially different from those encountered in the case of enolase and carboxypeptidase.

Carnosinase was chosen as the object of investigation because the crude preparations of carnosinase[1] showed practically all the characteristic features of peptidases, such as activation by only two metal ions, Mn^{2+} and Zn^{2+}, a lag period during the process of activation, instability of the enzyme solution, and stabilizing properties shown by Mn^{2+}. On the other hand a more refined procedure of purification[9] developed in this laboratory yields an apparently homogeneous protein material which under carefully defined conditions was stable for a period of several months.

MATERIALS AND METHODS

Purification of carnosinase

The preparation of carnosinase has been described earlier [9]. The purified enzyme was stored in solution at pH_5 7.70* in the presence of 0.01 *M* $MnCl_2$. The specific activity of the purified product from different preparations showed a maximal variation of approximately 10 %. The product from the first zone electrophoresis at pH_{20} 8.65 was used in preliminary measurements. The results were then checked with the enzyme of highest purity.

Reagents

The metal-ion solutions used were prepared from analytical grade salts, sulfates in all cases except $BaCl_2 \cdot 2H_2O$, $CaCl_2 \cdot 6H_2O$, $SrCl_2 \cdot 6H_2O$ and $HgCl_2$. Both $MnCl_2$ and $MnSO_4$ were used for the preparation of Mn^{2+} solutions, no differences being observed in the activating properties after changing the anion. The concentration of the $MnCl_2$ solutions was checked by analysis. The water used for dilution of the metal ion solutions and for preparation of buffers was distilled water that had been passed through a mixed-bed ion exchanger and stored in polyethylene bottles. The buffers used were prepared from chemicals of the highest available purity. Sigma 121 analytical grade tris(hydroxymethyl)aminomethane (THAM) was used for preparation of THAM–HCl, THAM-acetate and THAM–maleate buffers. As the pH of these buffers is strongly dependent on temperature, the pH values are stated with a suffix indicating the temperature at which the pH was measured.

The preparation and properties of carnosine (β-alanyl-L-histidine) have been described previously[9]. Glycyl-L-histidine was prepared according to the method of TURNER[11]. The melting point of the hydrochloride was 175°. Neither carnosine nor glycyl-L-histidine were free from traces of histidine. Comparison with a specially prepared and chromatographically pure carnosine sample showed no detectable difference for the kinetics of Mn^{2+}-activated hydrolysis. Both the substrate solutions and buffers within the pH region of 5–8 were purified by extraction with a 0.001 % solution of dithizone in CCl_4. The procedure, the efficiency and the pH limits of the extraction have been described by MALMSTRÖM[12]. Polyethylene bottles were used for storing the different buffers. In general, both in preparing the necessary solutions and in the analytical procedures described below, contamination by metal ions was minimized by the use of techniques described by THIERS[13] for work with trace elements.

Analytical methods

The determination of the protein content has been described earlier[9]. The total Mn content of the solutions was determined colorimetrically. The method used in the absence of chloride ions was the periodate method of SANDEL[14]. In the presence of chloride ions it was replaced by the persulfate method as described by NYDAHL[15]. In both cases a Beckman DU spectrophotometer was used for measuring the permanganate colour.

The free Mn^{2+} concentration was determined by measuring the electron spin resonance according to the method introduced by COHN[16]. The apparatus used was a Varian V-4500 EPR spectrometer with a rectangular H_{102} room temperature cavity. The temperature of the cavity was 36 ± 2° as measured by a thermocouple. The procedure for the resonance measurements and the calculations were the same as described by MALMSTRÖM, VÄNNGÅRD AND LARSSON[17], except that the measurements were carried out in quartz tubes. Solutions of known $MnCl_2$ content were used for calibration purposes.

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* The subscript indicates the temperature of the solution that was measured, with both the glass-electrode and the reference electrode at the temperature of the solution.

The concentration of free Cd^{2+} and free Zn^{2+} was determined according to the metal-indicator method described by RAAFLAUB[18]. Ammonium murexide was used as the indicator. In all cases the final murexide concentration was $1.5 \cdot 10^{-5}$ *M*. The measurements were performed at pH 7.65. A Beckman DU spectrophotometer with a thermostatically-controlled sample compartment was used for the measurements at different temperatures.

The pK values for metal–murexide complexes were determined at pH 7.65 and ionic strength 0.05. The pK of the Cd^{2+} murexide complex at 22° was 4.43. Fresh solutions of murexide were prepared for every series of measurements. A calibration curve of the extinction changes *vs.* metal ion concentration was then drawn and used for subsequent analysis of different solutions. The concentration range within which the determinations can be carried out was determined by the pK values of the metal–murexide complexes. Thus, Zn^{2+} concentrations lower than 10^{-5} *M* could not be measured. The same is valid for Cd^{2+} concentrations higher than 10^{-3} *M*.

Measurements of carnosinase activity

The amount of the substrate hydrolyzed was determined by withdrawing aliquots from the enzyme assay solution and titrating the liberated NH_2 groups in 90% ethanol according to GRASSMAN. The details of the procedure used have been described by SMITH[19]. In order to increase the reproducibility of the titration results, the visual end-point determination was replaced by a method utilizing photometric indication. The photometer assembly consisted of a filament light source and a photocell. The output from the photocell was then compared with a fixed voltage by means of a potentiometer using a light spot galvanometer as zero indicator. When thymolphtalein was used as the indicator the light source was equipped with a Hilger filter No. 606. A fixed arbitrary colour standard was used to calibrate the assembly before and between the measurements. Matched test tubes were used as titration vessels. Systematic investigations of the assay procedure showed that the relatively large error was due mainly to differences in the volume of the aliquot. The concentration of the substrate (0.05 *M*) was constant for all the measurements. The molarity of the buffers varied as they were prepared to give a constant ionic strength in the assay solutions. Detailed information on the composition of each solution is given in connection with the description of the separate experiments. The activity is expressed as moles of substrate split/min (*a*). The activity is determined from the linear part of the progress curve. No assumptions have been made as regards the order of the reaction.

Electrodialysis

The removal of the activating metal ions from the enzyme solutions was effectuated by means of electrodialysis against buffered solutions. The cell used, shown in Fig. 1, is a simple three compartment electrodialyser.

The two equal halves (A) of the cell made of Perspex (70 × 65 × 40 mm inner dimensions) with a double membrane* (C) between them, are held together by a metal frame (B). A suitably bent glass rod (D) (approximately 1–2 mm in diam.) is inserted between the two membranes to form a thin compartment for the protein solution to be dialyzed. The flow of fresh buffer through the electrode compartments containing Pt wire electrodes, takes place through PVC* tubes (E). The buffer in all the dialysis experiments was THAM–HCl, pH_5 7.70 and ionic strength 0.04. The voltage applied was 0.5–1.0 V/cm of the distance between the electrodes, which gave a current of 35–50 mA. A buffer flow of 0.5–1 l/h through each of the electrode compartments kept the pH and temperature of the protein solution steady. Fig. 2 shows a curve demonstrating the rate of dialysis achieved under the conditions specified. The concentration of the enzyme solution changes due to osmosis, but only in the case of concentrated enzyme solutions. As a rule the protein content was determined both before and after dialysis.

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* A section of Visking cellulose sausage casing purified according to HUGHES AND KLOTZ[20].

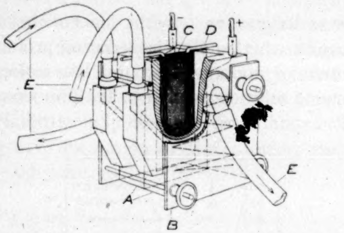

Fig. 1. Cell for electrodialysis. For description of details see text.

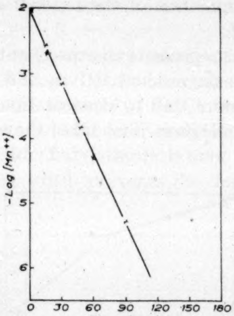

Fig. 2. Electrodialysis of 2 ml of 0.01 *M* $MnSO_4$ against THAM–HNO_3 buffer, pH_5 7.70, ionic strength 0.04. Voltage applied per cm of electrode distance, 1 V; current 35 mA.

EXPERIMENTAL

Effect of enzyme concentration on the activity

A necessary prerequisite for using activity values as a measure of the concentration of the active enzyme, is the existence of direct proportionality between the activity and the enzyme concentration. This must be the case under conditions where the concentration of the activating metal ion as well as the concentration of all the other substances capable of influencing the activity is kept constant. Activity as a function of carnosinase concentration was measured over the range of $0.6–6.0 \cdot 10^{-3}$ mg of protein N/ml of the assay solution. The variation in concentration was obtained by diluting a concentrated enzyme solution. The concentrated enzyme solution containing 0.01 *M* Mn^{2+} was dialyzed 48 h against a THAM–HCl buffer of pH_5 7.65 having the same Mn^{2+} concentration as the enzyme solution. The buffer was then used to dilute the enzyme solution. The plot obtained was linear.

Dependence of the enzymic activity on the presence of Mn^{2+} ions

Fig. 3 represents an electrodialysis experiment where the enzyme solution dialyzed originally contained 0.01 *M* Mn^{2+}. Aliquots of the dialysate were withdrawn after the indicated time intervals and one half of each aliquot was used to determine the residual activity at pH_{40} 7.65. The total Mn^{2+} concentration in the other half of each aliquot was restored to the value before electrodialysis (0.01 *M*) by addition of fresh Mn^{2+} and the activity determined under conditions identical with those used for determination of the residual activity. Total Mn^{2+} concentration in the assay: $2 \cdot 10^{-3}$ *M*.

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* Polyvinylchloride.

Fig. 4 represents an experiment which was designed to show the effect of chelating agents on the residual activity and to determine whether the losses during prolonged dialysis were due to denaturation of protein or contamination by low-molecular weight substances. Two ml of the same enzyme solution as used in the previous experiment were electrodialyzed under identical conditions. After 2 h of electrodialysis, 0.2 ml of EDTA was added as a 0.1 *M* solution, the pH_5 value of which was adjusted with NaOH to 7.70*. The electrodialysis was then continued for an additional 2 h after which the dialysate was removed and stored at the temperature of dialysis, while the activity measurements were continued after intervals indicated in the figure. At point A in Fig. 4, one-tenth volume of 0.1 *M* Mn^{2+} was added to one part of the dialysate. The activity in this new solution was then measured simultaneously with the measurements on the unaltered dialysate. The experiment represented by Fig. 4 was repeated with the activity measurements carried out at 10° instead of 40°. A six-fold higher enzyme concentration was used, with THAM–acetate pH_{10} 7.65 buffer instead of THAM–HCl. The activity changes during dialysis showed the same pattern as shown in Fig. 4.

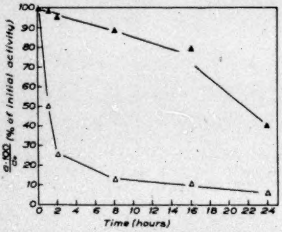

Fig. 3. Effect of removal of ions by electrodialysis on the activity of carnosinase. Two ml of a solution of carnosinase containing 0.01 *M* Mn^{2+} dialyzed against THAM–HCl buffer pH_5 7.70, ionic strength 0.04. Voltage 0.9 V/cm of electrode distance, current 35 mA. △—△, residual activity at pH_{40} 7.65; ▲—▲, activity after reactivation of the assay with $2 \cdot 10^{-3}$ *M* Mn^{2+} at pH_{40} 7.65.

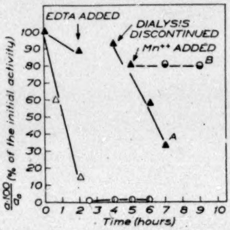

Fig. 4. The influence of EDTA on the electrodialysis of 2 ml of a solution of carnosinase, containing 0.01 *M* Mn^{2+}, against THAM–HCl buffer of pH_5 7.70, ionic strength 0.04. Voltage 0.9 V/cm of electrode distance, current 35 mA. △—△, residual activity measured at pH_{40} 7.65 before the addition of EDTA; ○—○, after the addition of EDTA; ▲—▲, activity after reactivation of the assay with $2 \cdot 10^{-3}$ *M* Mn^{2+}; ◒—◒, activity in a part of the dialysate stored after addition of Mn^{2+} (0.01 *M* Mn^{2+} in the solution).

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[*] The activity after the addition of EDTA was zero. Under the conditions employed this means that any possible activity higher than 0.5% of the initial value would have been measurable. The activity after the removal of EDTA varied between 2–6% of the value for the reactivated enzyme.

The following two experiments illustrate the influence of changing Mn^{2+} concentration on the time course of the hydrolytic reaction.

The experiment represented by Fig. 5 represents the case where the enzymic hydrolysis of carnosine in the presence of Mn^{2+} was suddenly interrupted by addition of EDTA (0.02 ml of 0.2 *M* EDTA to 2.5 ml of assay solution). Fifteen minutes later an excess of Mn^{2+} (0.05 ml of 0.2 *M* solution) was added in order to bind the EDTA and restore the free Mn^{2+} concentration. Curve B represents a control experiment performed with an unchanged part of the original assay solution. The three lines are drawn separately as the addition of EDTA and Mn^{2+} alters the titration values of the aliquots. The activity is not zero in the presence of EDTA because the amount of EDTA added had to be limited so that it could later easily be removed by addition of reasonable amounts of Mn^{2+}. High Mn^{2+} concentrations result in extensive formation of MnO_2 during the titration which prevents the detection of the end-point.

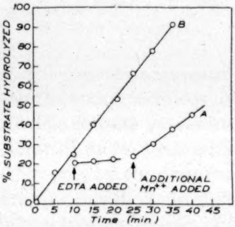

Fig. 5. Hydrolysis of carnosine as a function of time at pH_{40} 7.65. Initial total concentration of Mn^{2+}, $2 \cdot 10^{-3}$ *M*. Curve A represents the activity after the successive addition of EDTA ($1.6 \cdot 10^{-3}$ *M*) and Mn^{2+} ($4 \cdot 10^{-3}$ *M*), curve B the activity with no addition of any kind.

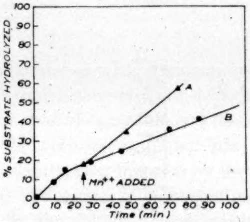

Fig. 6. Hydrolysis of carnosine as a function of time at pH_{40} 7.65. Curve A in presence of $3 \cdot 10^{-3}$ *M* Mn^{2+}; curve B in presence of approx. $5 \cdot 10^{-5}$ *M* Mn^{2+}.

The purpose of the experiment illustrated by Fig. 6 was to show that the results of the previous experiments were not due to reversible inhibition of the enzyme by EDTA. An incompletely dialyzed solution of the enzyme, with a residual activity of approximately 20 % of the initial value and a free Mn^{2+} concentration of approximately $5 \cdot 10^{-5}$ *M* was used for the assay (no EDTA was used during the dialysis). After the hydrolysis had proceeded for 10 min the assay solution was divided into two equal parts and an additional amount of Mn^{2+} was added to one of them (the final total concentration in this case being $2 \cdot 10^{-3}$ *M*). The velocity of hydrolysis in both halves of the solution is represented by curves A and B in Fig. 6.

Activation by different divalent metal ions

The effect of Ba^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Mn^{2+} on the rate of the enzymic hydrolysis of carnosine was investigated under the conditions described below. The activity measurements were in all cases conducted at 40°. THAM–HCl and THAM–maleate buffers were used to vary the pH between 5 and 9. As a rule the stable enzyme solution containing 0.01 *M* Mn^{2+} was dialyzed by the addition of EDTA as described in the preceding section. The dialyzed enzyme solution with a residual activity of approximately 5 % of the initial value was mixed with the buffer to be used. A suitable amount of a metal ion was added and the assay performed immediately. In order to ascertain the losses of activity during the dialysis* a control with Mn^{2+} as the activator was run for every dialysis. Table I summarizes the experimental conditions and the qualitative results of the activity measurements.

The activating effect of Cd^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} was studied as a function of the pH of the assay solution. Fig. 7 represents the activity curves obtained. THAM–HCl buffers were used between pH_{40} 7.5 and 8.6, THAM–maleate between 7.5 and 6. acetic acid–Na acetate below 6 and boric acid–Na borate above 8.6. The pH_{40} of the reaction mixture was determined with a pH meter in the absence of the enzyme.

TABLE I

METAL IONS TESTED AS ACTIVATORS FOR CARNOSINASE AT 40° AND A SUBSTRATE CONCENTRATION OF 0.05 *M*

Metal ion	*The concentration* range of the metal ion investigated* ($\times 10^{-3}$)	*Range of pH tested*	*Activity*
Ca^{2+}	2	7.5–9.0	—
Mg^{2+}	2–5	7.5–9.0	—
Ba^{2+}	2	7.5–8.65	—
Mn^{2+}	2	6.0–9.0	+++
Ni^{2+}	2–4	7.0–7.5	—
Co^{2+}	2	5.5–8.0	+
Fe^{2+}	1–2	5.0–7.0**	—
Zn^{2+}	2	5.0–8.0	++
Cd^{2+}	2	5.0–8.0	+++

* Refers to the total metal ion concentration.
** The concentration at higher pH values is nominal due to oxidation.

The ionic strength of the assay due to the buffer and metal ion was kept constant and equal to 0.056. The contribution from the 0.05 *M* substrate is not included but as the substrate concentration is constant the only variation of the ionic strength is due to the shift of the equilibrium between the different ionized forms of the substrate resulting from the changing pH. Due to the method used for activity determinations it is not possible to use buffer concentrations high enough to keep the pH of the reaction mixture constant. The pH changes after 50 % hydrolysis do not, however, exceed 0.1 unit. The total metal-ion concentration was in all the different measurements equal to $2 \cdot 10^{-3}$ *M*. The free metal-ion concentration varies from case to case depending on the stability constants of the metal complexes with the different molecules and ions of the solution. The concentrations of the enzyme solutions used for the different measurements were all identical before the electrodialysis. Due to variations in activity losses during the dialysis, the specific activity values of the dialyzed solutions vary from 5 to 25 %. In order to utilize data from several experiments for the same group of curves the activity of every dialysate is determined at pH_{40} 7.65 in the presence of $2 \cdot 10^{-3}$ *M* Mn^{2+}. The activity at this arbitrarily chosen point is represented by 100 and the results of the other activity determinations expressed relative to it.

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* The losses during the dialysis varied between 10–25 %. In the case of the losses exceeding 30 % the enzyme solution was discarded.

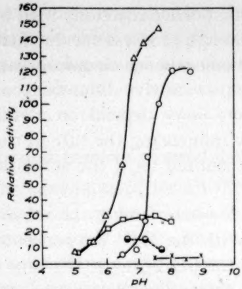

Fig. 7. Effect of pH on the hydrolysis of carnosine at 40° in presence of different metal ions: ○—○, $2\cdot10^{-3}M$ Mn^{2+}; △—△, $2\cdot10^{-3}M$ Cd^{2+}; □—□, $2\cdot10^{-3}M$ Zn^{2+}; ◒—◒, $2\cdot10^{-3}M$ Co^{2+}; ×—×, residual activity after electrodialysis of the Mn^{2+}–carnosinase.

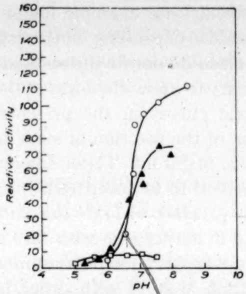

Fig. 8. Effect of pH on the hydrolysis of glycyl-L-histidine at 40° in the presence of different metal ions: ○—○, $2\cdot10^{-3}$ M Mn^{2+}; ▲—▲, $2\cdot10^{-3}$ M Cd^{2+}; □—□, $2\cdot10^{-3}$ M Zn^{2+}.

Fig. 8 represents analogous curves of pH dependence with the same metal ions and under the same conditions, but with carnosine replaced by glycyl-L-histidine as the substrate. The activity curves in this case, as in the case of carnosine, are discontinued at the point of metal hydroxide formation, as the activity at higher pH values is affected not only by the diminishing metal-ion concentration but also by inactivation of the enzyme due to co-precipitation with metal hydroxide. It is interesting to note that Mn^{2+} activation is only slightly influenced by the change of the substrate. The maximum activity obtained with the same total concentration of Cd^{2+} and Zn^{2+} diminishes considerably. This is not unexpected as the replacement of an β-amino group by an α-amino group increases the binding of Cd^{2+} and Zn^{2+} to the substrate and as a result, among other possible changes influencing the activity, the free metal-ion concentration diminishes as compared to the assay with carnosine. Some relative values are given in Table II.

TABLE II

THE EFFECT OF DIFFERENT SUBSTRATES ON THE CONCENTRATION OF FREE METAL ION AT 20°, pH 7.65

Metal ion	*Total conc. ($\times 10^{-3}$) M*	*Buffer (0.05 M)*	*Free Me^{2+} conc. in presence of*	
			0.05 M carnosine	*0.05 M glycyl-L-histidine*
Cd^{2+}	2	THAM–maleate	$5\cdot10^{-5}$	$2.8\cdot10^{-5}$
Zn^{2+}	2	THAM–HCl	$9.5\cdot10^{-4}$	$4.5\cdot10^{-4}$*

* The error in determinations of free Zn^{2+} can be rather large due to possible interference by $Zn(OH)_2$.

Progress curves of the enzyme reactions in the presence of different metal ions

Before a further comparison can be made between the effects of different metal ions on the enzymic reaction it is necessary to study the time course of the enzymic reaction in the presence of different metal ions. It is by no means necessary that the progress of the reaction is of the same type for all the activating ions. SMITH AND

SPACKMAN[2] have for example found both zero and first-order reactions with leucine aminopeptidase depending on the activating ion. Although progress curves of enzyme reactions often do not fit the standard equations of homogeneous chemical reactions, it is necessary to know the form of the progress curves, as relative differences between the progress curves in the presence of different ions may depend on a different mechanism of the reaction or some factor selectively influencing the rate in the presence of one metal ion. These differences must be accounted for if the activation by different ions is to be compared.

In the presence of Mn^{2+} ions in the pH region between 7 and 9, the substrate is hydrolyzed in accordance with zero order kinetics. With the Mn^{2+} ion concentration lower than necessary for maximum activity or with the pH below 7, the time curve diverges from that of zero order (see Fig. 6). As these divergences are strongly influenced by the temperature of the assay mixture, they can be explained by the fact that the stability of the enzyme is a function of the Mn^{2+} concentration and the pH of the assay. Figs. 9 and 10 show the progress curves of the reaction in the presence of Cd^{2+} and Zn^{2+} at an initial concentration giving maximal activity. In both cases the curves diverge considerably from the zero order curve. Two of the possible factors responsible for the deviation can easily be derived from the experimental data. The first factor reveals itself in the sharp change of the free metal-ion concentration during the progress of the reaction, especially striking in the case of Zn^{2+} activation. The downward slope of the curve representing the free metal-ion concentration is determined by the difference in metal binding capacity between the substrate and the products of the reaction.

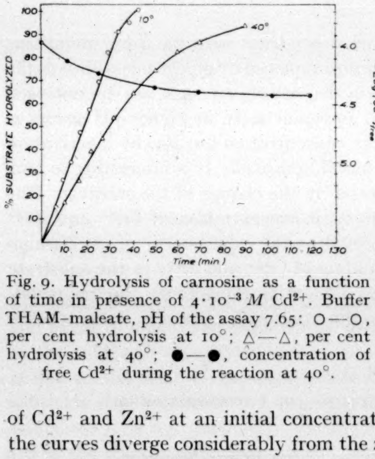

Fig. 9. Hydrolysis of carnosine as a function of time in presence of $4 \cdot 10^{-3}$ M Cd^{2+}. Buffer THAM–maleate, pH of the assay 7.65: ○—○, per cent hydrolysis at 10°; △—△, per cent hydrolysis at 40°; ●—●, concentration of free Cd^{2+} during the reaction at 40°.

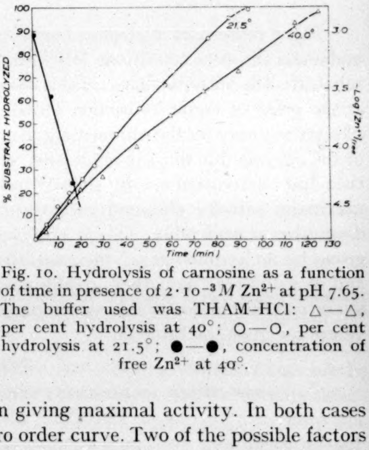

Fig. 10. Hydrolysis of carnosine as a function of time in presence of $2 \cdot 10^{-3}$ M Zn^{2+} at pH 7.65. The buffer used was THAM–HCl: △—△, per cent hydrolysis at 40°; ○—○, per cent hydrolysis at 21.5°; ●—●, concentration of free Zn^{2+} at 40°.

The presence of a second factor is clearly indicated by the temperature dependence of the shape of the progress curves. In both cases, though more drastically in the case of Cd^{2+}, the downward curvature of the reaction curve diminishes when the reaction is carried out at lower temperatures.

The time course of the activation process

As the enzyme was stored in the presence of Mn^{2+}, the lag period observed by

HANSON AND SMITH[1] showing the time dependence of the activation process would naturally not show up. In the case of activation by Cd^{2+} and Zn^{2+} of the dialyzed solutions, no lag period was observed. A more detailed investigation of this question will be published in connection with investigations of metal-ion stabilization of carnosinase, which are now in progress.

Activity as a function of metal ion concentration

The enzyme used for all the measurements described in this section originated from the same concentrated solution of enzyme in THAM–HCl buffer of pH_5 7.70 containing 0.01 M Mn^{2+}. The measurements in the presence of Mn^{2+} were carried out at a constant enzyme concentration obtained by dilution of the stock solution. The activity measurements were carried out immediately after dilution. For measurements in the presence of Cd^{2+}, suitable amounts of the stock solution were dialyzed as described before. The dialyzed solution was immediately used for activity measurements. Separate dialyses were carried out for each series of activity measurements. The activating metal ions were added to the assay immediately before the addition of the enzyme. The buffer used for measurements in the presence of Mn^{2+} was THAM–HCl. When the temperature was lowered, the content of THAM had to be reduced considerably in order to keep the pH at 7.65 and to retain the same ionic strength. At lower temperatures the buffer capacity of THAM–HCl is very small in the region of pH 7.65. Thus already at 10° the pH is buffered by the substrate–HCl system instead.

THAM–maleate buffers of the same ionic strength were used for measurements in the presence of Cd^{2+} ions added in the form of $CdSO_4$. The Cl^- ions were deliberately omitted in order to avoid complex formation between Cd^{2+} and the excess of chloride ions. The free metal-ion concentration was in the case of Mn^{2+} determined as described in the section devoted to methods. As the measurements could be carried out at 36° only, the values given at other temperatures are subject to an error, the magnitude of which depends on the temperature dependence of the metal binding capacity of the substrate. From the available data on the metal binding capacity of imidazole[21, 22], it can be estimated that the temperature ought not to influence the order of magnitude of the free Mn^{2+} concentration.

Direct studies of the chelate formation between the substrate and the activating metals now in progress will furnish the necessary corrections. The influence of temperature on the Mn^{2+} binding by THAM molecules has been neglected as the pK value of the THAM–Mn^{2+} complex cannot be higher than 1.1[23]. In the case of Cd^{2+}, the concentration is determined at the different temperatures used for activity measurements.

Fig. 11 shows the influence of Mn^{2+} concentration on the activity of carnosinase. The range of the metal-ion concentration was in this case, and in the case of Cd^{2+} limited by the method of activity determinations. At higher metal-ion concentrations, the formation of metal hydroxide precipitates disturbs the titration. Fig. 12 shows the same type of curves in the presence of Cd^{2+}.

An attempt can be made to plot the data in terms of the simple empirical rate equation of WARBURG AND CHRISTIAN[24],

$$a = \frac{a_{max} M}{K_a + M} \qquad (1)$$

where a represents activity and M the concentration of the free metal ion, conveniently used in the form introduced by LINEWEAVER AND BURK[25]. K_a represents a hypothetical dissociation constant.

$$\frac{1}{a} = \frac{K_a}{a_{max}} \frac{1}{M} + \frac{1}{a_{max}} \qquad (2)$$

The experimental points from the experiments with Mn^{2+} at 20°, 30° and 40° do not yield straight lines. Fig. 13 shows the form of the LINEWEAVER–BURK function at 30°. The results from measurements at 10.5° fit reasonably well on a straight line.

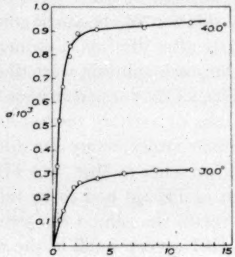

Fig. 11.

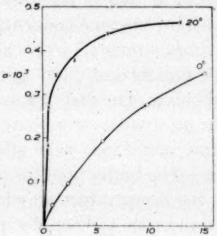

Fig. 12.

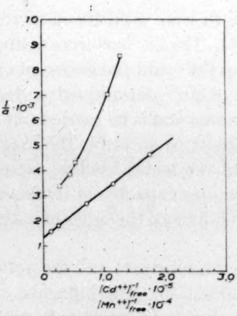

Fig. 13.

Fig. 11. The influence of the concentration of Mn^{2+} on the activity, a, of carnosinase in THAM-HCl buffer at 30° and 40°, pH 7.65.

Fig. 12. The influence of the concentration of Cd^{2+} on the activity, a, of carnosinase in THAM–maleate buffer at 20° and 0°, pH 7.65.

Fig. 13. LINEWEAVER–BURK plots, eq. (2), for the activation of carnosinase by: □—□, Mn^{2+} at 30° in THAM–HCl buffer; ○—○, Cd^{2+} at 12° in THAM–maleate buffer.

The measurements in the presence of Cd^{2+} at 2°, 12° and 20° all yield straight lines when plotted according to LINEWEAVER AND BURK. Fig. 13 shows the plot at 12°. Due to the well-known insensitivity of the plotting at high metal-ion concentrations the apparent linearity does not prevent the K_a values from varying within approximately 20% when calculated from the experimental points. The K_a values for Cd^{2+} measurements at different temperatures are given in Table III. The K_a values for Mn^{2+} activation at 20°, 30° and 40° are replaced by limits of variation of the constant for the unaltered Warburg equation.

Inhibition of Mn^{2+} carnosinase by metal ions

The determination of the inhibitory properties of metal ions on the Mn^{2+}-activated

TABLE III

THE VALUES FOR CONSTANT K_a (EQ. 1) FOR Mn^{2+} AND Cd^{2+} ACTIVATION AT pH 7.65

Metal ion	K_a *values at different temperatures*				
		10.5°	*21°*	*30°*	*40°*
Mn^{2+}		$8.0 \cdot 10^{-4}$	$3–1 \cdot 10^{-4}$	$1.5–0.4 \cdot 10^{-4}$	$0.6–0.3 \cdot 10^{-4}$
	2°	*12°*	*21°*		
Cd^{2+}	$1.2 \cdot 10^{-4}$	$1.5 \cdot 10^{-5}$	$5.0 \cdot 10^{-6}$		

carnosinase furnishes us with an additional experimental parameter, namely the classification of metal ions with respect to their relative abilities to inhibit the enzyme. The information obtained from such studies has been used to draw conclusions about the groups participating as ligands in the binding of the activating ion to the assumed site of catalytic action.

A more strict investigation on the quantitative aspects of inhibition characterized by determination of the competitive or non-competitive character of the inhibition has not been carried out in the case of carnosinase. This is due to the fact that an investigation of a system with two different metal ions both interacting with the substrate, necessitates the determination of substrate–metal association constants for all the metal ions involved. A qualitative survey of the inhibiting effects of some divalent metal ions has been carried out prior to a more thorough investigation.

The measurements were carried out at pH_{40} 7.65 with THAM–HCl as the buffer. The concentration of Mn^{2+} and of the metal studied was in all cases 0.002 M.

These concentrations were for some of the ions investigated (Fe^{2+}, Hg^{2+}) only nominal, representing the total amount of the metal ion added. The results obtained are presented in Table IV.

TABLE IV

INHIBITION (IN %) OF CARNOSINASE ACTIVITY (100% = TOTAL INHIBITION) IN THAM–HCl BUFFER AT pH 7.65 AND 40° IN THE PRESENCE OF 2×10^{-3} M Mn^{2+} AND 2×10^{-3} M OF THE INHIBITING ION

Metal ion	*Inhibition (%)*	*Metal ion*	*Inhibition (%)*	*Metal ion*	*Inhibition (%)*
Be^{2+}	86	Sr^{2+}	0	Co^{2+}	80
Mg^{2+}	0[a]	Ba^{2+}	0	Fe^{2+}	64
Ca^{2+}	0[b]	Zn^{2+}	85	Ni^{2+}	65
		Cu^{2+}	0	Hg^{2+}	40

[a,b] In addition measurements were carried out at pH_{40} 8.65–9.10 which showed no inhibition.

DISCUSSION

Activation and stabilization

In order to show that an observed dependence of activity on the presence of metal ions is not due to several possible artifacts[10] but to the fact that the metal ion functions as a coenzyme, it is necessary, as a first step, if the enzyme solution contains metal ions, to show that their removal is followed by loss of activity which can be restored by adding suitable amounts of the activating ion.

If the stability of the enzyme molecule allows the successive removal of the metal ions by prolonged dialysis against chelating agents, the experiments can easily be performed. If there is reason to suspect that the metal-free enzyme is unstable, it is essential that the rate of dialysis should considerably exceed the rate of the subsequent denaturation. For carnosinase preliminary experiments showed that dialysis, even in its most effective form, always resulted in considerable activity losses. Electrodialysis described in the section for methods was, however, found to satisfy the requirements for the rate of dialysis. The pH used for the dialysis is very near the iso-electric point of the enzyme as determined by qualitative electrophoretic experiments. Experiments with pH values lower or higher than this invariably led to precipitation of the protein on the membranes.

The first part of the curve, Fig. 3, representing activity changes during dialysis shows a rapid decrease in the residual activity. This process is reversible, as reactivation restores about 90 % of the original activity. After 3 h, the rate of the dialysis has decreased so far that further decrease of the residual activity takes place only very slowly and is accompanied by a gradually increasing amount of irreversible inactivation indicated by the downward slope of the reactivation curve. The drop of activity might perhaps be caused by accumulation of strongly inhibiting ions from the buffer used for dialysis. It is clear that the residual activity after 2 h of dialysis can scarcely be cut down further by prolonged dialysis. Even if the inactivation could be avoided the dialysis is bound to lead to an equilibrium where the removal of ions is compensated by ions from the continuously changing buffer. The proportions of the dialysate (2 ml) and the buffer (5.1 l) make this feasible even if the buffer has been prepared with the utmost care.

Experiments with the use of chelating agent show that EDTA completely inhibits the activity. The residual activity after the removal of EDTA by electrodialysis is about 2 % of the initial value. The continuous decrease of activity, even after the dialysis has been discontinued, shows that the losses of activity during dialysis are due to irreversible inactivation which proceeds at a considerable rate* even at 5°. The addition of Mn^{2+} to a part of the dialysate (A in Fig. 4) immediately stopped the inactivation. The most important consequence of this surprisingly rapid inactivation is that no definite conclusion can be drawn about the role of the Mn^{2+} ion. The observed activation can be explained as stabilization in view of the fact that the dialysis takes place at 5° and the activity measurements at 40°. The metal-free enzyme is probably denatured so fast at 40° that the activity is entirely determined by the amount of enzyme stabilized by the remaining Mn^{2+} ions. In order to prove that, beside its role as a stabilizer, the Mn^{2+} does function as a true activator it will be necessary to show that the metal-free enzyme cannot function as a catalyst during the short period before it is irreversibly inactivated.

A repetition of the electrodialysis with the activating measurements performed at 10° instead of 40°, however, confirmed the observations of the preceding experiment.

As the validity of this kind of experiment depends on the relative rates of the supposed catalytic action of the metal-free enzyme and the inactivation process, two additional experiments were designed to give more experimental material before drawing any definite conclusion.

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* The rate of denaturation varied considerably in different electrodialysis runs although the same enzyme solution was used. Before the process of denaturation has been studied systematically, the data given should be considered only as a qualitative indication of the rate of denaturation.

In both cases the time course of the reaction is studied when the Mn^{2+} concentration in the assay is rapidly changed during the reaction.

In the case of experiments with EDTA (Fig. 5), straight lines representing the time course of the reaction are found and would be expected if Mn^{2+} functions as a true activator independently of the stabilization phenomenon. In the next experiment the addition of Mn^{2+} to a reaction mixture of low Mn^{2+} content results in a considerable increase of activity clearly distinguished from a possible stabilization effect. That the first part of the time course of this reaction is non-linear is explained by the rapid inactivation of the metal-free enzyme which directly influences the equilibrium: $Mn^{2+} + E^n \rightleftharpoons MnE^{n+2}$. MnE^{n+2} represents here the stable enzyme and not the assumed active enzyme–coenzyme compound, the site for the metal ion in the stabilization process not necessarily being identical with the enzymically active site. The results of the series of experiments described above lead to the conclusion that the Mn^{2+} ions in the reaction medium have two functions to fulfil. In the first place, they are indispensable for the catalytic activity of the enzyme, as is characteristic of metal-ion-activated enzymic reactions. These data alone naturally do not allow one to conclude whether the metal ion participates directly in the catalytic reaction or not. The second function of the metal ion is the stabilization of the enzyme molecule. This aspect of the Mn^{2+}–enzyme interaction can scarcely be compared to the frequently observed, rather unspecific, influence of metal ions on the thermal stability of proteins. In these cases the proteins are as a rule stable without the metal ion under normal conditions, and the stabilization reveals itself as a decrease in the rate of denaturation at higher temperatures[26–28]. The rapid inactivation of metal-free carnosinase at 5° and full stability in the presence of Mn^{2+} at temperatures as high as 40° make it plausible that the stabilization is due to specific interaction between the enzyme and one or more metal ions; a similar situation is found in the case of Ca^{2+} ion stabilization of amylase[29].

The metal-ion specificity of the activation and inhibition

If we compare the metal ions that have been shown to activate carnosinase with respect to the physical properties generally used to characterize ions, it becomes obvious that the electro-negativities of the activating ions lie very close to each other and consequently, when the well-known order of complex stability[30] is considered the activating ions all fall together in the middle of a series $Hg^{2+} > Cu^{2+} > Ni^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+} > Cd^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$ listed according to decreasing complex stability with amino acids.

TABLE V

SOME PROPERTIES OF THE ACTIVATING METAL IONS[10]

Metal ion	*Electronegativity*	*Ionic radius*	*Covalent radius*
Mn^{2+}	1.4	0.78–0.80	—
Cd^{2+}	1.5	0.96	1.48 tetrahedral
Zn^{2+}	1.5	0.72–0.74	1.31 tetrahedral
Co^{2+}	1.7	0.73	1.23 octahedral

The comparison of the maximal activities obtained with different ions is rather difficult as the time course of the hydrolytic reaction shows considerable differences in the presence of the different activating ions. The product formation curves in the presence of Cd^{2+} and Zn^{2+} indicate the possibilities of product inhibition and enzyme denaturation. Due to the insensitivity of the activity measurements, the real initial velocities may differ considerably from the values estimated from the curves since the first experimental point often represents as much as 10 % hydrolysis.

If we consider the pH region of activation for different ions, it is easy to realize that the activity obtained for example in the presence of Co^{2+} need not represent the maximal possible activity in the presence of free metal ions in concentrations equal to the total amount of metal ion added. Oxidation of the Co^{2+}–substrate compound manifested by strong colour formation in the assay solution can, by altering the free metal-ion concentration, also produce chelated forms that act as inhibitors. The decisive influence of the interaction of metal ions with the substrate molecules is further illustrated by Fig. 8 which shows the pH dependence of the metal-ion activation with carnosine replaced by glycyl-L-histidine. The relative activities obtained with the same nominal metal-ion concentration as was used in the case of experiments with carnosine show quite a different pattern.

The specific inhibition of Mn^{2+} activated carnosinase by metal ions can advantageously be discussed in this connection as both the specificity of activation and inhibition are largely determined by the same factors. The most interesting feature of the results from inhibition studies is the strong inhibitory effect shown by Be^{2+}, especially when compared to the other alkaline-earth ions which show no inhibitory tendencies at all. Pronounced inhibition by Be^{2+} has in the case of the phosphatases[31] been interpreted as indicating the participation of a phenolic –OH in the formation of the binding site for the metal ion. Before any such suggestion can finally be accepted it should be remembered that Be^{2+} differs radically from all other divalent ions with respect to its size. The small size of Be^{2+} may enable the ion to block some necessary negatively charged groups (not necessarily phenolic –OH) in the interior of the protein structure which are not available for other larger divalent ions.

The strong inhibiting properties of Zn^{2+} and Co^{2+} are expected since both ions can function as activators. The apparent non-inhibiting properties of Cu^{2+}, which are peculiar when compared to the inhibitory effect shown by Ni^{2+}, are explained easily enough in terms of the chelate-forming ability of the substrate. The pK values of Cu–carnosine[32] indicate that in this case the substrate acts as a chelating agent comparable to EDTA so that the concentration of metal ions free to react with the protein is negligible.

When correlating the physical properties of the metal ions with the results of the activity and inhibition measurements it must be remembered that the observed specificity need not only reflect the spatial and electronic requirements of the active site for catalytic action but can be determined by a number of factors influencing the free metal-ion concentration of the reaction medium.

Generally speaking the factors determining the metal ion specificity can be divided into two groups:

A. Factors influencing the equilibrium in the assay solution between the metal ions and the active site.

B. Factors directly connected with the physical properties of the metal ion and of the group or groups that combine with the metal ion in the course of the catalytic reaction.

The first group of factors do not influence the intrinsic binding constant of the metal ion to the active site but rather influence the concentrations of the different molecular species participating in the enzyme-catalyzed reaction (*e.g.*, chelate formation between the substrate or the product and the metal ions, formation of metal hydroxides, enzyme denaturation and instability of the oxidation state of the metal ion.) The free metal-ion concentration is not only diminished by direct removal of ions but in addition the formation of mixed compounds of the type enzyme-Me-$(OH)_n$ or substrate-Me-$(OH)_n$ can inhibit the reaction.

For the second group it should be possible to find a correlation between the physical properties of the metal ion and the degree of activation, as the factors of this group can directly be studied by physical measurements of different, separated and well-defined molecules acting as models for the enzyme reaction.

It is in principle not impossible that the observed specificity should be entirely determined by the secondary factors listed in the first group. In the case of carnosinase practically all the factors of the first group seem to influence the results; thus it is very doubtful if a significant pattern can emerge from the correlation of activity data with the properties of the metal ion. The one definite conclusion that can be drawn is that the previously assumed high specificity of carnosinase is misleading and is due to difficulties in obtaining a stable metal-free enzyme and to not taking necessary precautions to control the secondary factors indirectly influencing the activity (for example enzyme denaturation).

Kinetics of metal-ion activation

The Cd^{2+} activation of carnosinase can adequately be described by the simple mass-action equation of WARBURG AND CHRISTIAN[24]. The experimental values plotted according to LINEWEAVER AND BURK yield straight lines. The values of the constant K_a in eq. (2) are listed in Table IV. The conclusion from these data is that one metal ion is necessary for every active site. In the concentration range investigated no other function can be discovered that influences the activity and is dependent on metal-ion concentration. Inhibition at higher metal-ion concentrations cannot be discovered from the activity curves. The results naturally give us no information as to whether the enzyme is stable or whether an independent process of inactivation is taking place. The temperature dependence of the time course of the reaction indicates that although Cd^{2+} activates the enzymic reaction it does not necessarily stabilize the enzyme structure in the way that Mn^{2+} ions do.

The Mn^{2+} activation curves at room temperature (20°) and higher do not fit the WARBURG–CHRISTIAN equation, and as can be seen from Fig. 13 a LINEWEAVER–BURK plot does not yield a straight line.

The WARBURG–CHRISTIAN mass action equation can naturally be modified by the introduction of an additional parameter to give an equation that fits the experimental points of the Mn^{2+} activation curves better. Eq. (3) for example adequately describes the functional dependence of the activity (a) on the free Mn^{2+} concentration (M).

$$a = \frac{a_{max}M}{B + \frac{A}{M} + M} \tag{3}$$

The need for a modification in the empirical rate equation can be deduced from the assumption that an inactivation process dependent on Mn^{2+} concentration takes place. Observations on the instability of the metal-ion free enzyme and the stabilizing role of Mn^{2+} make it apparent that inactivation at low metal-ion concentration must be included in the deduction of a rate equation.

Let us assume that after the lowering of the metal-ion concentration the process of inactivation, leading to different forms of the enzyme, arrives at a state of apparent equilibrium. This means that further changes in the relative amounts of the different forms of the enzyme are slow when compared to the hydrolysis of the substrate. Further, if we assume that the equilibrium with the metal ion determining the rate of hydrolysis is identical with the stabilization equilibrium (the same metal ion functions in both capacities), it is easy to see that the rate equations for the reaction are of the same type as eq. (1), regardless of the number of steps postulated for the inactivation process. It is assumed that activation and stabilization, both influencing the total activity measured, are dependent on the equilibrium (4) where ME designates the stable

$$K = \frac{ME}{M_{free} \times (E_{tot.} - ME)} \tag{4}$$

active form of the enzyme. The introduction of different forms of inactive enzyme E' combined or uncombined with the metal ion leads only to a lowering of the free enzyme concentration represented by the additional terms in eq. (5). Consequently, when

$$K = \frac{ME}{M_{free} \times (E_{tot.} - ME - E'M - E')} \tag{5}$$

the two assumptions concerning equilibrium conditions are fulfilled, the LINEWEAVER–BURK plots should yield straight lines independent of the number of forms of the inactivated enzyme present.

The concave curve representing the LINEWEAVER–BURK plot for Mn^{2+} activation is of the type expected for a second order reaction. If we assume that activation and stabilization are represented by two different and independent equilibria with different dissociation constants, K_1 and K_2, the rate eq. (6) arrived at is analogous to the

$$\frac{V_m}{V} = 1 + \frac{K_1}{M} + \frac{K_2}{M^2} \tag{6}$$

equation derived by FRIEDENWALD AND MAENGWYN-DAVIES[35] for cases where the same substance acts as both activator and substrate. Eq. (6) is identical with the empirical rate eq. (3). In deducing eq. (6) we assume the existence of an activated enzyme species $EM_{act.}$ that is not stabilized but can combine with the stabilizing atom to form $EM_{2stab.act.}$. On the contrary, if we assume that the enzyme must be stabilized before it can be activated, $EM_{act.}$ can be assumed to be non-existent, and the rate equation will be further simplified. Which of the two equations represents the experimental curves better cannot be decided as differences between the two theoretical curves lie within the limits of error for the activation measurements. The theoretical curve for Mn^{2+} activation at 30° according to eq. (7) coincides with

$$\frac{V_m}{V} = A + \frac{K_1 K_2}{M^2} \tag{7}$$

the curve drawn in Fig. 11. In the case of activation at 40°, the experimental curve differs very slightly at the two points representing the lowest Mn^{2+} concentration. In this case eq. (6) gives a somewhat better result.

There may be a second explanation for deviations from the linearity for the LINEWEAVER–BURK plot. The condition assumed for deducing the rate expressions, namely the existence of an equilibrium between the different forms of the enzyme, may not be fulfilled. Experimental evidence on this point is not sufficient yet. We know that the inactivation is not totally reversible, which should naturally exclude any possibility of equilibrium. On the other hand the progress curve of the reaction (Fig. 6) which represents the activity at low metal-ion concentrations, shows that, after a period of decreasing activity, the system seems to arrive at a sort of equilibrium where the rate of further denaturation of the enzyme is so low that for calculations during a reasonable time interval it can be considered as being in equilibrium. A more detailed study of inactivation is necessary before the kinetic data can definitely be interpreted.

The conclusion that can definitely be drawn is that Mn^{2+} activation cannot be represented by a simple mass law treatment and that the simplest way to explain it, is to assume two different equilibria with the Mn^{2+} ion, the first for stabilization and the second for activation. This adequately explains the concave curve of the simple LINEWEAVER–BURK plot. It does, in addition, account for the observed effect of inactivation during the course of the other experiments described. The data are not sufficient yet to say whether the number of active sites and the number of ions per enzyme molecule necessary for stabilization is equal as has been tacitly assumed for calculating the rate equations.

The strong temperature dependence of the K_a values is rather interesting as the metal-ion binding to proteins is reported to be very little influenced by temperature changes[34]. As the constant K_a represents an algebraic combination of a number of equilibrium constants, including the acid dissociation constants of the groups responsible for binding the metal ion to the enzyme or to the substrate, the thermodynamic quantities calculated cannot be directly correlated to any possible activation mechanism. However, the apparent values can possibly be used to differentiate between different possible rate equations when the temperature dependence of the constants included in K_a can be determined separately. In the case of the substrate, this certainly is feasible and studies on this question are being planned. Thus, the findings from the kinetics of metal-ion activation confirm the results from the other experiments and emphasize the dual role of metal ions played in the case of carnosinase. It is by no means evident that the metal-ion specificity for activation is the same as for stabilization; if this is not the case, the total or apparent specificity is a combination of the influences imposed by a pair of metal ions.

As regards the nature of the activation process less can be said. The equilibrium seems to be established fast enough, in the case of Cd^{2+} and Zn^{2+}, to prevent any type of lag period being observed with the rather insensitive methods used for activity measurements. It is possible that the lag period in the activation by Mn^{2+} ions observed by previous workers[1] is connected with the stabilizing properties of this ion. The investigation of this problem is in progress and will be published separately. A more general discussion of the data presented, in the light of current concepts of the mechanism of peptidases, will be given in a subsequent communication describing the stabilization of enzymes by metal ions.

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DIE HEMMUNG DER AKTIN–L-MYOSIN INTERAKTION IN LEBENDEN UND EXTRAHIERTEN MUSKELN DURCH UREA

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SUMMARY

The inhibition of the actin–L-myosin interaction by urea

1. With increasing concentration, urea transforms actomyosin ATPase of washed myofibrils and of purified actomyosin gels into L-myosin ATPase. This transformation is completed as soon as the urea concentration has risen to about 1 *M*. This transformation is reversible.

2. Up to a urea concentration of 1 *M* the activity of L-myosin ATPase is not affected. The inefficacy of 1 *M* urea on the activity of L-myosin ATPase has been determined with L-myosin gels and L-myosin sols in the presence of Ca and Mg.

3. With a further increase in the urea concentration up to 2 *M* a slight inactivation of L-myosin ATPase takes place. This inactivation appears to be completely or to a large extent irreversible.

4. The action of acetamid is similar to that of urea. The interaction of actin and L-myosin, however, is not completely inhibited until acetamid reaches a concentration of 2 *M*, whereas L-myosin ATPase apparently begins to be impaired by acetamid when the concentration of acetamid exceeds 1 *M*.

5. Also the mechanical interaction of actin and L-myosin filaments is inhibited by 1 *M* urea in the presence of ATP. The ATP-contraction of extracted fibres is followed by relaxation on the addition of 1 *M* urea and the resistance to stretch falls to values similar to zero.

6. Living muscles in 1 *M* urea solutions no longer respond mechanically to electrical stimulation. This inhibition is fully reversible. The analogous inhibition by means of 1 *M* acetamid is incomplete. 2 *M* acetamid causes complete inhibition, which is only partially reversible.

7. Living muscles in 1 *M* urea solutions loose only a small amount of water, whereas urea rapidly penetrates into the interior of the fibre until complete equilibrium of the urea concentration has been reached.

8. 1 *M* urea within living muscle fibres does not affect the ATP turnover of resting living muscle. 1 *M* urea, however, strongly decreases ATP splitting if the living muscle is induced to an increased chemical and mechanical activity by contracture substances or other contracture producing factors.

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9. The chemical and mechanical inhibition of muscular activity is not based on the effect of urea on the excitation of the membrane. Resting potential as well as action potential remain practically unchanged in the presence of 1 *M* urea.

10. All experimentally tested forms of muscle contracture (produced by eleven different contracture substances) are completely inhibited by 1 *M* urea. As far as was experimentally determined, this inhibition is reversible. This result is independent of whether the contractures are accompanied by a depolarization of the membranes or take place in depolarized muscles.

11. All experiments show that apparently in living muscle, too, the activity of urea attacks the last common link of the reaction chains which lead to contraction or contractures. This last common link is apparently the interaction between actin filaments, L-myosin filaments, and ATP.

EINLEITUNG

Es ist seit langem bekannt, dass die Kohäsionskräfte zwischen Aktin und L-Myosin auch bei niedrigen Ionenstärken verschwinden, wenn ATP anwesend ist, aber nicht gespalten wird. So sinkt der statische Dehnungswiderstand extrahierter Muskelfasern auf ~ Null, wenn in ATP-haltigen Lösungen die ATP-Spaltung durch Salyrgan vergiftet wird[1]. In diesem Fall wird die ATP-Spaltung durch Vergiftung der L-Myosin-ATPase verhindert. Kürzlich wurde gezeigt, dass auch durch eine bestimmte Gruppe von Substanzen, die Interaktions-Inhibitoren, in Anwesenheit von ATP komplette Dissoziation der Aktin- und L-Myosin-Filamente in der extrahierten Muskelfaser erreicht werden kann, obwohl diese Interaktions-Inhibitoren die Aktivität der L-Myosin-ATPase überhaupt nicht beeinflussen[2]. Zur Gruppe der Interaktions-Inhibitoren gehören ausser dem Erschlaffungsfaktor[3] eine Reihe Substanzen von bekannter chemischer Konstitution.

Die Interaktions-Inhibitoren eröffnen durch die eigenartige Spezifität ihrer Wirkung die Möglichkeit, den Mechanismus der Muskel- und Aktomyosinkontraktion in verfeinerter Weise zu analysieren. Denn die Kontraktion beruht offensichtlich auf der Interaktion zwischen Aktin- und L-Myosin-Filamenten[4,5]. Es erscheint deshalb nützlich die Liste der chemisch bekannten Interaktions-Inhibitoren zu verlängern um zu sehen, welche chemische Eigenschaften allen Interaktions-Inhibitoren gemeinsam sind. Die bisher bekannten Interaktions-Inhibitoren sind teils Kationen, teils Anionen[2,6]. Einige von ihnen tragen viele Ladungen (Polylysin, Protamin, Polyäthensulfonat, Fuadin, EDTA) andere tragen nur eine Ladung (SCN).

Es wird in dieser Arbeit gezeigt, dass auch Nicht-Elektrolyte als Interaktions-Inhibitoren wirken können.

Die interaktionshemmenden Nicht-Elektrolyte Urea und Acetamid sind ausserdem so klein-molekular, dass sie auch in lebenden Muskeln eindringen. Infolgedessen kann gezeigt werden, dass diese Interaktions-Inhibitoren in lebenden Muskeln ebenso wirken wie in Aktomyosingelen, gewaschenen Fibrillen und Wasser-Glyzerin extrahierten Fasern.

I

Bei einer Ionenstärke von 0.08 μ wird die ATP-Spaltung durch Mg aktivierte Aktomyosin-Systeme mit steigender Urea-Konzentration stark gehemmt (Fig. 1 und

2). Sowohl in natürlichen und künstlichen Aktomyosinglen wie in gewaschenen Fibrillen beträgt die ATPase Aktivität in Anwesenheit von 1.0–1.5 M Urea nur noch zwischen 10–20 % der unbeeinflussten Aktivität. Das bedeutet, die ATPase-Aktivität ist unter 1.0–1.5 *M* Urea in allen Mg aktivierten Aktomyosin Systemen vom Wert der Aoktmyosin-ATPase auf den Wert der L-Myosin-ATPase abgesunken (Fig. 1 Kurve 3 und 4, Fig. 2 Kurve 2). Bei weiterer Erhöhung der Urea-Konzentration sinkt die Spaltungsgeschwindigkeit des ATP deutlich aber nur unwesentlich noch weiter ab.

Dagegen beeinflusst die Anwesenheit von Urea die ATP-Spaltung durch Sole und Gele von reinem L-Myosin weder bei Ca-Aktivierung noch bei Mg-Aktivierung bis zu einer Urea-Konzentration grösser als 1 *M* (Fig. 3). Hierin verhält sich Urea

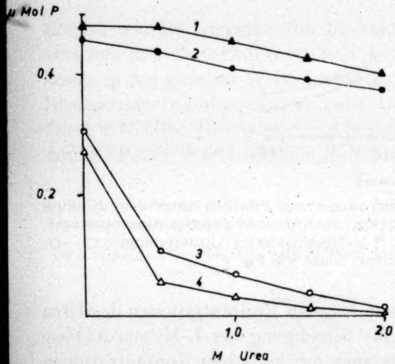

Fig. 1. Die ATP-Spaltung durch künstliches und natürliches Aktomyosingel unter dem Einfluss von Urea (pH, 7.0; Temperatur, 22°; μ, 0.08; $5 \cdot 10^{-3}$ *M* ATP). Abszisse: Molarität des Urea. Ordinate: Spaltungsgeschwindigkeit in µMol/mg Eiweiss/min. ▲—▲, durch 10^{-2} *M* Ca-aktiviertes künstliches Aktomyosingel; ●—●, 10^{-2} *M* Ca-aktiviertes natürliches Aktomyosingel; ○—○, $5 \cdot 10^{-3}$ *M* Mg-aktiviertes natürliches Aktomyosingel; △—△, $5 \cdot 10^{-3}$ *M* Mg-aktiviertes künstliches Aktomyosingel.

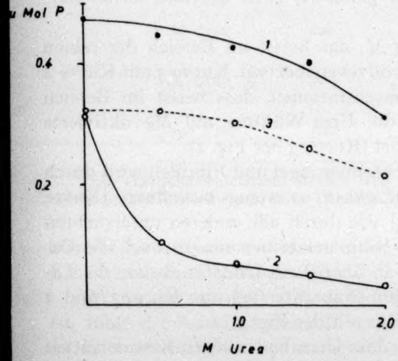

Fig. 2. Die ATP-Spaltung durch Fibrillen unter dem Einfluss von Urea. ●—●, 10^{-2} *M* Ca-Aktivierung; ○—○, $5 \cdot 10^{-3}$ *M* Mg-Aktivierung; ○ - - ○, $5 \cdot 10^{-3}$ *M* Mg-Aktivierung nach Auswaschen des Urea. Sonst wie Fig. 1.

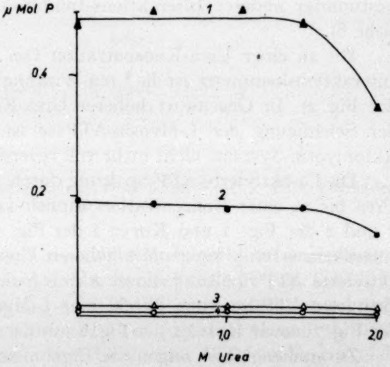

Fig. 3. Die ATP-Spaltung durch L-Myosingel ($\mu = 0.08$) und L-Myosinsol ($\mu = 0.56$) unter dem Einfluss von Urea. ▲—▲, 10^{-2} *M* Ca-aktiviertes L-Myosingel; ●—●, 10^{-2} *M* Ca-aktiviertes L-Myosinsol; △—△, $2.5 \cdot 10^{-3}$ *M* Mg-aktiviertes L-Myosingel; ○—○, $2.5 \cdot 10^{-3}$ *M* Mg-aktiviertes L-Myosinsol; $5 \cdot 10^{-3}$ *M* ATP bei Ca-aktivierung; $2.5 \cdot 10^{-3}$ *M* ATP bei Mg-Aktivierung. Sonst wie Fig. 1.

wie alle anderen bisher untersuchten Interaktions-Inhibitoren, die frei von Nebenwirkungen sind (z.B. Erschlaffungsfaktor, Polyäthensulfonat, EDTA, Protamin). In noch höheren Konzentrationen ($\sim$ 2 *M*) wird auch die Aktivität der L-Myosin ATPase deutlich herabgesetzt (Fig. 3). In diesen höheren Konzentrationen des Urea wird die Hemmung der Interaktion durch eine Schädigung der L-Myosin-ATPase überlagert. Ähnliche Nebenwirkungen wurden auch mit zu hohen Konzentrationen bestimmter anderer Interaktions-Inhibitoren gefunden (z.B. Polyanetholsulfonat; siehe 6).

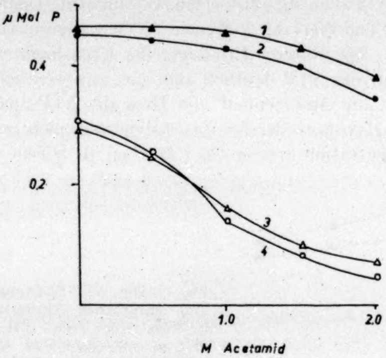

Fig. 4. Die ATP-Spaltung durch natürliches Aktomyosingel und Fibrillen unter dem Einfluss von Acetamid. Abszisse: Molarität des Acetamid. ▲—▲, 10^{-2} *M* Ca-aktiviertes Aktomyosingel; ●—●, 10^{-2} *M* Ca-aktivierte Fibrillen; △—△, $5 \cdot 10^{-3}$ *M* Mg-aktiviertes Aktomyosingel; ○—○, $5 \cdot 10^{-3}$ *M* M g-aktivierte Fibrillen. Sonst wie Fig. 1.

Bis zu einer Urea-Konzentration von 1 *M*, das heisst im Bereich der reinen Interaktionshemmung, ist die Urea-Wirkung voll reversibel (vgl. Kurve 3 mit Kurve 2 der Fig. 2). In Gegenwart höherer Urea-Konzentrationen, dass heisst im Bereich der Schädigung der L-Myosin-ATPase ist die Urea-Wirkung auf Mg aktivierte Aktomyosin-Systeme nicht mehr voll reversibel (Kurve 3 der Fig. 2).

Die Ca-aktivierte ATP-Spaltung durch Aktomyosingel und Fibrillen wird durch Urea bis zu einer Konzentration ähnlich 1 *M* genau so wenig beeinflusst (Kurve 1 und 2 der Fig. 1 und Kurve 1 der Fig. 2) wie durch alle anderen untersuchten Interaktions-Inhibitoren[6]. Mit höheren Urea-Konzentrationen nimmt auch die Ca-aktivierte ATP-Spaltung durch Aktomyosin in ähnlichem Umfang ab wie die Ca-aktivierte ATP-Spaltung durch reine L-Myosin-Präparate (vgl. die Kurve 1 und 2 der Fig. 1, sowie Kurve 1 der Fig. 2 mit der Kurve 1 der Fig. 3).

Zusammengefasst zeigen die Ergebnisse, dass Urea bis zu einer Konzentration ähnlich 1.0 *M* ein Interaktions-Inhibitor ohne Nebenwirkungen ist, und dass bei einer Konzentration des Urea ähnlich 1 *M* die enzymatische Interaktion zwischen Aktin und L-Myosin vollständig gehemmt ist.

Auch Acetamid erniedrigt die ATP-Spaltung durch Mg-Aktivierte Fibrillen und Aktomyosingele auf ungefähr den Wert der L-Myosin-ATPase. Doch wird dieser Betrag der Hemmung, erst mit 2 *M* Acetamid erreicht (Kurven 3 und 4 der Fig. 4). Auf der anderen Seite wird die Ca-aktivierte ATP-Spaltung von Fibrillen und Aktomyosingelen durch 2 M Acetamid in ähnlichem Umfang herabgesetzt (Kurven 1 und 2 der Fig. 4), wie durch 2 M Urea. Der Acetamid-Einfluss auf reines L-Myosin ist nicht untersucht. Trotzdem sehen diese Beobachtungen so aus als wäre auch Acetamid ein Interaktions-Inhibitor, der schwächer wirkt als Urea, während die Nebenwirkungen etwa die gleichen sind. Infolgedessen wird durch 1 M Acetamid die Interaktion noch keineswegs vollständig aufgehoben, während unter 2 M Acetamid bereits deutliche Nebenwirkungen vorliegen. Zu dieser Annahme passt die Tatsache, dass die Wirkung einer 2 M Acetamidlösung nicht mehr voll reversibel ist (vgl. Abschnitt III).

II

Ebenso wie die enzymatische Interaktion wird auch die mechanische Interaktion zwischen den Aktinfilamenten und den L-Myosinfilamenten der extrahierten Psoasfasern in der gleichen Weise beeinflusst, wie durch die anderen bisher untersuchten Interaktions-Inhibitoren. Der hohe Dehnungswiderstand der starren Fasern wird durch 1 M Urea überhaupt nicht beeinflusst, aber durch Zusammenwirken von Urea und ATP auf Werte ähnlich Null herabgesetzt (Tabelle I). Die Nebenwirkungen höherer Urea-Konzentrationen (2 M) sind angedeutet in einer sehr geringfügigen Erniederung des Dehnungswiderstandes auch in Abwesenheit von ATP (vgl. Tabelle I).

TABELLE I

DEHNUNGSWIDERSTAND VON EXTRAHIERTEN EINZELFASERN IN ANWESENHEIT VON UREA

pH, 7.0; 0.05 M KCl; $3 \cdot 10^{-3}$ M $MgSO_4$; Temperatur, 22°.

	Ausgangslange $(\frac{L}{L_0} \times 100)$	*Statischer Dehnungswiderstand* $(g \times cm^{-2} \times L \times \Delta L^{-1})$		*Zahl der Versuche*
		Mit ATP $3 \cdot 10^{-4}$ M	*Ohne ATP*	
1 M Urea	50	~ 0*		4
1 M Urea	100		6,600 7,200	2
2 M Urea	100	~ 0*	5,200 4,500	4
Ohne Urea	100		6,500	1

* Ein Dehnungswiderstand ähnlich Null ist kleiner als 150 g × $cm^{-2} \times L \times \Delta L^{-1}$. Für andere methodischen Einzelheiten, siehe TECHNISCHER TEIL.

Wenn eine extrahierte Psoasfaser durch $3 \cdot 10^{-3}$ M ATP zur Kontraktion gebracht ist, führt der Austauch des ATP gegen 1 bis 2 M Urea (vgl. Fig. 5a und 5b) nicht zu einer wesentlichen Spannungsabnahme, weil Urea ohne ATP die Fasern nicht weich macht, sondern in kontrahiertem Zustand erstarren lässt. Auf Zusatz von $3 \cdot 10^{-3}$ M ATP zu dem ureahaltigen Bad tritt dagegen sofort die rapide und vollständige Erschlaffung ein, die auf Grund des Absinkens des Dehnungswiderstandes auf Null zu erwarten ist (vgl. Fig. 5a und 5b). Diese Erschlaffung in Gegenwart von Urea ist ebenso reversibel, wie die Erschlaffung durch alle anderen Interaktions-Inhibitoren[2]. Dass Urea allein eine kontrahierte Faser nicht oder kaum zur Erschlaffung bringt während Urea und ATP zusammen Erschlaffung hervorrufen ist zuerst von Bozler beobachtet worden[7]. Die hohe Wirksamkeit der Kombination von Urea und ATP ist ausserdem von Hoffmann-Berling zur Verhinderung bestimmter Zellbewegungen in Glyzerin-extrahierten Zellen benutzt worden[8].

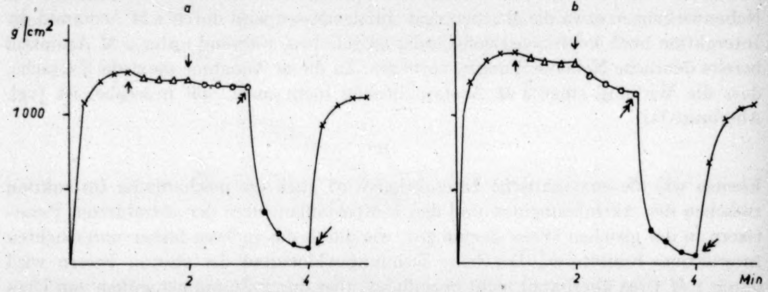

Fig. 5. Erschlaffung der kontrahierten Fasern durch Urea und ATP. ($3 \cdot 10^{-3}$ *M* $MgSO_4$; $5 \cdot 10^{-2}$ *M* KCl; 10^{-2} *M* PO_4; pH, 7.0; Zimmertemperatur; Anfangslänge = 33 % der Standardlänge; Faserdurchmesser: (a) = 40 μ, (b) = 50 μ). Abszisse: Zeit; Ordinate: Spannung. ×—×, $3 \cdot 10^{-3}$ *M* ATP. ↑, Auswaschen des ATP; ↓, (a) 1 *M* Urea; (b) 2 *M* Urea; ↟, (a) 1 *M* Urea + $3 \cdot 10^{-3}$ *M* ATP; (b) 2 *M* Urea + $3 \cdot 10^{-3}$ *M* ATP; ↡, $3 \cdot 10^{-3}$ *M* ATP.

III

Werden lebende Muskeln in eine Ringer-Lösung gebracht, die ausserdem eine 1 *M* Konzentration an Urea enthält, so dringt das Urea ausserordentlich schnell in die einzelnen Muskelfasern ein (Tab. II). Infolgedessen wird das Konzentrationsgleichgewicht zwischen "aussen" und "innen" in erster Linie durch Diffusion und nicht durch osmotischen Wasserentzug von Muskelwasser erreicht. Der osmotische Wasserverlust beschränkt sich auf die ersten Minuten der Urea-Anwesenheit, weil nur während dieser ersten Minuten der Konzentrationsgradient des Urea an der

TABELLE II

DIFFUSION UND WIRKUNG DES UREA IM LEBENDEN MUSKEL

Belastung der Muskeln: Sartorius 3 g; Rectus 7 g; Gastrocnemius 15 g. Erklärung siehe im Text; Methodische Einzelheiten siehe TECHNISCHER TEIL.

	Zeit in Minuten nach Zugabe des Urea	*Kontraktilitat in % der Kontraktilitat vor Zugabe des Urea*	*Molare Urea Konzentration*					
			im Gesamtvolumen des Muskels	*im Wasser der Muskelfasern*				*im Muskelbad*
				Unterer Grenzwert		*Oberer Grenzwert*		
				Unkorrigiert	*Korrigiert*	*Unkorrigiert*	*Korrigiert*	
	(1)	*(2)*	*(3)*	*(4)*	*(5)*	*(6)*	*(7)*	*(8)*
(1) Sartorius	1.5	7	0.38	0.40	0.43	0.49	0.51	0.91
	7.0	0	0.69	0.90	0.98	0.90	0.96	0.89
(2) Rectus	2.0	40	0.23	0.18	0.18	0.30	0.30	0.90
	10.0	0	0.42	0.47	0.49	0.55	0.57	0.93
(3) Gastrocnemius	5.0	78	0.15	0.04	0.04	0.19	0.19	0.92
	30.0	0	0.39	0.43	0.43	0.51	0.51	0.88

Membran steil ist. Mit dem Konzentrationsausgleich des Urea kehrt das Gewicht langsam wieder zum normalen Gewicht zurück. Der maximale Wasserverlust tritt bei kleinen Muskeln früher ein und ist grösser als bei grossen Muskeln, weil in sehr kleinen Muskeln die Oberfläche der einzelnen Fasern ziemlich unmittelbar mit der 1 *M* Urea-Lösung in Berührung kommt. Infolgedessen ist der Konzentrationsgradient des Urea an der Faseroberfläche und infolgedessen auch der osmotische Wasserverlust hier verhältnismässig gross. Auf der anderen Seite führt der steile Konzentrationsgradient zu einem schnellen Urea-Durchtritt durch die Membran und damit zu einem frühen Konzentrationsausgleich zwischen Faserinnern und Bad (vgl. Tab. II). In grossen Muskeln grenzt die Oberfläche der meisten Fasern an den extracellulären Raum, in den das Urea nur langsam hineindiffundiert und aus dem es gleichzeitig fortlaufend in die Fasern eindringt. Infolgedessen ist der Konzentrationsgradient an der Oberfläche der Mehrzahl der Fasern in dicken Muskeln viel kleiner. Dies führt zum verminderten osmotischen Wasserverlust und zu einem langsameren Konzentrationsausgleich zwischen Faserinnerem und Bad. Infolgedessen verlieren sehr kleine Muskeln von 50 mg (Sartorien) in Maximum des Wasserverlustes etwa 30 % ihres Anfangsgewichtes, Muskeln von 140 mg (Recti) 20 % und Muskeln von 325 mg (Gastrocnemii) nur 10 % des Anfangsgewicht. Das Minimum des Muskelgewichtes (Maximum des Wasserverlustes) wird bei den 50 mg Muskeln nach 5 Min, bei den 140 mg Muskeln nach 10 Min, und den 325 mg Muskeln nach etwa 20 Min erreicht.

Durch einen 20 %igen osmotischen Wasserverlust wächst die Salzkonzentration im Innern der Fasern auf das 1.45fache, mit 10 %igem osmotischen Wasserverlust auf das 1.19fache, falls das "freie" Muskelwasser nach HILL[9] zu 77 % und des auf den extracellulären Raum fallende Wasser nach CONWAY[10] als 13 % des Muskelgewichtes angenommen wird. Da in dieser Arbeit für die Beobachtungen des Urea Einflusses auf lebende Muskeln, Sartorien und Recti, von einem Gewicht von über 100–500 mg verwendet werden, dürfte sich die Salzkonzentration "innen" aus osmotischen Gründen höchstens auf das 1.5fache und meist weniger erhöht haben. Diese Erhöhung der Salzkonzentration bleibt weit hinter den Salzkonzentrationen zurück, die in den Arbeiten von HILL[11], HOWARTH[12], HODGKIN UND HOROWICZ[13] die maximale Spannung und maximale Arbeit der Muskelzuckung herabsetzen.

Die Wirkungen von Urea und Acetamid sind also offenbar direkte Folgen des Eindringens dieser Substanzen in die Muskelfaser und nicht Folgen der damit verbundenen Erhöhung der Salzkonzentration "innen". Hierfür spricht auch die Tatsache dass die Wirkungen von 1 *M* Acetamid und 1 *M* Urea sehr verschieden sind obwohl ihre osmotischen Wirkungen auf die Salzkonzentration "innen" völlig gleich sind (Fig. 6).

[redacted]

Fig. 6. Vergleich der Kontraktionshemmung eines Rectusmuskels (465 mg) mit Urea und Acetamid in Ringer-Lösung. Osmotischer Gewichtsverlust durch Urea oder Acetamid $< 5\%$. Abszisse: Zeit, Reizintervall 5 Sec. Ordinate: Hubhöhe bei Belastung mit 15 g. Zunächst Reizung in Ringer-Lösung, dann ↓, Austausch gegen 1 *M* Urea enthaltende Ringer-Lösung; ↑, Auswaschen des Urea mit Ringer; ↓↓, 1 *M* Acetamid in Ringer; ↑, Auswaschen des Acetamid; ↓↓↓, 2 *M* Acetamid in Ringer; ↑, Auswaschen des Acetamid.

Die Urea-Konzentration innerhalb der Fasern kann berechnet werden aus der Urea-Konzentration, die im Gesamtvolumen des Muskels analytisch bestimmt ist (Spalte 3 der Tabelle II). Das Ergebnis der Berechnung hängt ab von dem Anteil des Urea-lösenden Wassers im Innern der Fibrille am Gesamtgewicht des Muskels, sowie von der Urea-Konzentration im extracellulären Wasser des Muskels und seinem Volumenanteil. Für die Urea-Konzentration im extracellulären Wasser wird einmal angenommen, sie sei der Konzentration im Wasser des Faserinnern gleich. Unter dieser Annahme ergibt sich der obere Grenzwert der Urea-Konzentration im Faserwasser. Für die Annahme des unteren Grenzwertes ist angenommen, dass die Urea-Konzentration in extracellulärem Wasser und im Muskelbad gleich sei. Der Volumenanteil des extracellulären Raums ist mit 0.13 des Muskelgewichtes angesetzt[10]. Dann ergeben sich die Werte, die in Tabelle II als unterer und oberer Grenzwert "unkorrigiert" (Spalte 4 und 6) aufgeführt sind, falls der Anteil des Urea lösenden Muskelwassers (intracellulär + extracellulär) nach HILL mit 0.77 angesetzt wird[9].

Bei dieser Berechnungsart ist nicht berücksichtigt, dass der Anteil des extracellulären Raumes und des von Eiweiss erfüllten "Nichtlösenden Raumes" am Gesamtmuskel unter Urea vergrössert ist, weil das intracelluläre Wasser und damit auch der Gesamtmuskel durch osmotische Schrumpfung abgenommen haben, während das extracelluläre Wasser und der "Nichtlösende Raum" bei osmotischer Schrumpfung unverändert bleiben. Wenn die durch osmotische Schrumpfung hervorgerufenen Änderungen im Verhältnis des intracellulären Wassers zum extracellulären Wasser und zum "Nichtlösenden Raum" in Rechnung gestellt werden (siehe TECHNISCHER TEIL), ergeben sich die Werte unterer und oberer Grenzwert "korrigiert" (Spalten 5 und 7 der Tabelle II). Die Werte aller vier Spalten unterscheiden sich nicht allzu stark; doch dürften die "korrigierten" Grenzwerte der Wahrheit näher kommen.

Die Ergebnisse zeigen, dass bereits nach 7 Min die Urea-Konzentration im Innern der Fasern eines Sartorius (von 100 mg) der Konzentration im Bad vollständig gleich ist (Versuch 1 der Tabelle II). Bereits nach 1.5 Min ist die Urea-Konzentration im Mittelwert aller Fasern etwa halb so hoch, wie die Konzentration im Bad (Versuch 1 der Tabelle II). Mit zunehmender Dicke der Muskeln (Muskulus rectus, rund 230 mg und Muskulus gastrocnemius, rund 470 mg) nimmt die Zeit bis zum Konzentrationsausgleich erheblich zu. Mit dem M.rectus (Versuch 2 der Tabelle) werden 10 Min benötigt und mit dem M.gastrocnemius (Versuch 3 der Tabelle) sogar 30 Min, bis die Innenkonzentration ungefähr auf 0.5 *M* Urea angestiegen ist. Die ausserordentlich starke Zunahme der Zeit, die für den Konzentrationsausgleich benötigt wird, mit der Dicke des Muskels zeigt, dass die Ausgleichszeit mehr durch die Länge des Diffusionsweges als durch die Verlangsamung der Diffusion während des Durchtritts durch die Muskelmembran bestimmt ist.

Die Wirkung des Urea auf die Kontraktion des lebenden Muskels ist gross; immer dann, wenn die Urea-Konzentration im Innern der Faser einen Wert $\sim 0.5\, M$ erreicht hat, verschwindet die Kontraktion auf übermaximalen elektrischen Reiz vollständig (Tabelle II).

Das Verschwinden der Kontraktion ist zu erwarten, weil durch Urea-Konzentrationen zwischen 0.5 *M* und 1 *M* die Interaktion der Aktinfilamente und L-Myosinfilamente aufgehoben wird (vgl. Abschnitte I und II). Ebenso wie die Aufhebung der

Interaktion zwischen Aktin und L-Myosin ist auch die Kontraktionshemmung des lebenden Muskels, die durch 1 *M* Urea bewirkt wird, voll reversibel (Fig. 7).

Ebenso wie die Interaktion wird auch die Kontraktion des lebenden Muskels durch 1 *M* Acetamid nur unvollständig, dagegen durch 2 *M* Acetamid vollständig aufgehoben (Fig. 6). Die Hemmung der Vital-Kontraktion durch 2 *M* Acetamid ist nur noch teilweise reversibel. Dies passt zu der Tatsache, dass 2 *M* Acetamid zusätzlich zu der reversiblen Interaktionshemmung auch eine Schädigung der Ca-aktivierten ATPase herbeiführt (vgl. Abschnitt I).

[redacted]

Fig. 7. Reversible Kontraktionshemmung eines Sartoriusmuskels (153 mg) durch 1 *M* Urea in Ringer-Lösung. Abszisse: Zeit, Reizintervall 5 Sec. Ordinate: Hubhöhe bei Belastung mit 10 g. ↓, Austausch gegen eine 1 *M* Urea enthaltende Ringer-Lösung; ↑, Auswaschen des Urea mit Ringer-Lösung.

IV

Die Wirkung des Urea auf die Kontraktion ist auch im lebenden Muskel mit einer Hemmung der enzymatischen Interaktion zwischen Aktin- und L-Myosin-Filamenten verbunden. Kontrakturen des lebenden Muskels durch Chloroform, Avertin und Coffein führen bei Zimmertemperatur zu einer schnellen Abnahme der ATP-Konzentration (siehe z.B.[14]). Wird den fraglichen Lösungen aber 1 *M* Urea zugesetzt, so unterbleibt nicht nur die Kontraktur, sondern auch die Abnahme der ATP-Konzentration wird stark verlangsamt (Tabelle III).

Wenn dagegen der Muskel in Ruhe bleibt, wird der ATP-Spiegel durch die Anwesenheit von 1 *M* Urea nicht messbar beeinflusst (Tabelle IV). Ebensowenig

TABELLE III

EINFLUSS VON 1 *M* UREA AUF DIE MIT KONTRAKTUREN VERBUNDENE ATP-SPALTUNG LEBENDER MUSKELN (*M. rectus*).

Zimmertemperatur; Kontrakturstoffe und Urea sind in Ringer, pH 7.0, gelöst; Belastung 25 g; Vorbehandlung der Muskeln ohne oder mit Urea 15 Min unter O_2-Durchperlung, Kontraktur ohne O_2-Durchperlung.

	Kontraktur			*ATP-Gehalt**	
Durch	*Dauer in Minuten*	*Mechanische Arbeit in g × cm/g Muskel*		*in μMol/g Muskel*	
		Ohne Urea	*Mit Urea*	*Ohne Urea*	*Mit Urea*
(1) Chloroform gesättigt	5	29.7	0	0.86	1.52
(2) Avertin 0.5 %	15	41.4	0	0.62	1.56
(3) Coffein 0.3 %	30	50.0	0	0.19	1.28
(4) Coffein 0.3 %	45	53.3	0	0.00	1.11

* Analysiert mit Säulenchromatographie, methodische Einzelheiten siehe TECHNISCHER TEIL.

wird die Einbaurate von [^{32}P] anorganischem Phosphat, das dem Muskelbad zugefügt ist, durch die Anwesenheit von Urea verändert. ATP isoliert vom Urea behandelten ruhenden Muskel gibt 5,890 Impulse/Min/μMol und ATP vom Kontrollmuskel 5,760 Impulse (für Einzelheiten siehe TECHNISCHER TEIL). Auch der Gehalt des lebenden ruhenden Muskels an Spaltprodukten des ATP scheint in Gegenwart und Abwesenheit von Urea gleich zu sein (Tabelle IV).

TABELLE IV

NUCLEOTIDGEHALT IN UREAHALTIGEN UND UREAFREIEN RUHENDEN MUSKELN (M. RECTUS* UND M. SARTORIUS*)

Zimmertemperatur; 1 *M* Urea in Ringer gelöst.

Vorbenhandlung der Muskeln 15 Min unter O_2-Durchperlung	*μMol/g Muskel*				
	ATP	*ADP*	*AMP*	*IMP*	*Inosin*
Mit Urea	2.13	0.69	0.30	0.27	0.88
Ohne Urea	2.09	0.70	0.26	0.30	0.84

* Die Rectus- und Sartorius-Muskeln werden zusammen papierchromatographisch analysiert, (methodische Einzelheiten, siehe TECHNISCHER TEIL). Rectus/Sartorius Gewichtsverhältnis rund 2.5. Der ATP-Gehalt der Rectusmuskeln ist geringer. Wir finden in zwei gesonderten Versuchen mit der Säulenchromatographie μMol ATP/g Rectus:

	Ohne Urea	Mit Urea
(1)	1.49	1.67
(2)	1.43	1.38

TABELLE V

EINFLUSS VON 1 *M* UREA AUF DIE ATP-SPALTUNG NACH AUFTAUEN LEBEND GEFRORENER MUSKEL (M. RECTUS)

Nach dem Auftauen stehen die Muskeln 10 Min bei 0°. Erklärung siehe im Text. Analysiert mit Säulenchromatographie, methodische Einzelheiten, siehe TECHNISCHER TEIL.

Behandlung der Muskeln vor dem Gefrieren: O_2-Durchperlung 15 Min bei 22°	*μMol/g Muskel*					
	ATP	*ADP*	*AMP*	*Adenosin*	*IMP*	*Inosin*
Mit Urea	1.29	0.51	0.40	0.33	0.58	0.66
Ohne Urea	0.57	0.76	0.59	0.35	0.94	0.65

Wenn dagegen dem ruhenden ureahaltigen und ureafreien Muskel nach dem Gefrieren in flüssiger Luft Gelegenheit gegeben wird, wieder aufzutauen und vor der Analyse 10 Min bei 0° in aufgetautem Zustand zu bleiben, so ergibt sich ein ganz anderes Bild (Tabelle V). In dem ureafreien Muskel findet während dieser 10 Min der rapide ATP-Abbau statt, der die Taukontraktur gefrorener Muskel zu begleiten pflegt[15, 16].Im Muskel der 1 *M* Urea enthält findet in der gleichen Zeit nur ein bescheidener ATP-Abbau statt. Vollständige Hemmung des Abbaus ist auch durch Urea nicht zu erwarten, weil dieser Interaktions-Inhibitor weder die L-Myosin-ATPase noch die Grana-ATPase inaktiviert.

V

Der lebende ureahaltige Muskel verhält sich chemisch und mechanisch genau so, wie es auf Grund der Reaktion zwischen Urea und den isolierten kontraktilen Strukturen zu erwarten ist. Die gleiche Hemmung der Kontraktion und der ATP-Spaltung müsste aber auch auftreten, wenn die Erregbarkeit der Muskelmembran durch Urea aufgehoben würde. Wie weit Letzteres der Fall ist, ergibt sich aus der Höhe des Membranpotentials und Aktionspotentials. Beide Potentiale können gleichzeitig nur an Einzelfasern gemessen werden. Da die Kontraktion als maximale mechanische Spannung des Gesamtmuskels bestimmt wurde, wurden in jedem Zustand des Muskels die Membran- und Aktionspotentiale mehrerer Einzelfasern gemessen. Wenn nach 5 Min der Einwirkung von 1 *M* Urea der Ausschlag des Mechanogramms verschwunden ist, zeigt sich in der Höhe des Ruhepotentials und des "spike" keine wesentliche Änderung (vgl. Fig. 8a mit 8b). Nur das Ruhepotential ist um 5 mV erniedrigt*.

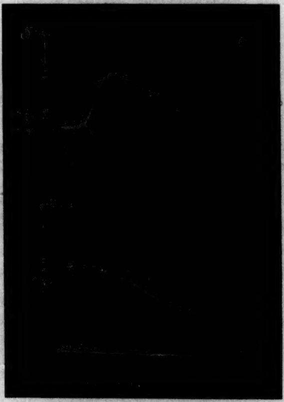

Fig. 8. Aktionspotential und Mechanogramm eines Sartoriusmuskels (a) in Ringer-Lösung (oberes Bild), (b) 5 Min in einer 1 *M* Urea enthaltenden Ringer-Lösung (unteres Bild). Abszisse: Zeit. Ordinate: links; mV des Membranpotentials, rechts; mechanische Spannung. Das Mechanogramm oben zeigt einen isometrischen Spannungsgipfel von etwa 12 g etwa 15 mSec nach dem Reiz. Die Potentialkurve zeigt vor dem "spike" des Aktionspotentials eine kleine "spike"-ähnliche Zacke, die auf dem Einbruch des Reizstroms beruht. Im unteren Bild verläuft die Spannungskurve völlig parallel zur Abszisse auf einen Spannungswert ähnlich 0.1 g, der durch die Vorspannung des ruhenden Muskels gegeben ist. Die Amplitude der elektrischen Potentialkurve ist trotz des Fehlens einer mechanischen Antwort von normaler Grösse.

Dieser Befund zeigt, dass die Urea-Wirkung an dem lebenden Muskel nicht aus einer Änderung der Membran-Erregbarkeit erklärt werden kann.

Durch welche Mechanismen im lebenden Muskel die Erregung der Membran mit der Aktivierung des kontraktilen Systems verbunden ist, ist noch sehr wenig bekannt. Wie kompliziert die Situation ist, ergibt eine Betrachtung der Liste der kontrakturerzeugenden Substanzen. Unter den Kontrakturstoffen, die nicht in den Muskel eindringen, finden sich Stoffe, die ähnlich, wie die physiologische Membran-Erregung, nur durch Depolarisierung polarisierter Muskelmembranen flüchtige Kontrakturen erzeugen; ferner finden sich Stoffe, die Dauerkontrakturen erzeugen mit oder ohne gleichzeitige Depolarisation der Membran; schliesslich finden sich Stoffe, wie Coffein, die auch an depolarisierten Membranen genau die gleiche Kontraktur hervorrufen, wie an polarisierten Membranen. In der Tabelle VI sind 11 solcher Kontrakturstoffe aufgeführt und gleichzeitig zeigt Tabelle VI, dass durch 1 *M* Urea alle diese Kontrakturen aufgehoben werden. Die Tabelle zeigt ferner, dass immer dann, wenn der Kontrakturstoff eine Dauerkontraktur macht, die durch Urea zunächst gehemmte Kontraktur nachgeholt wird, sobald das Urea ausgewaschen ist.

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* Über die elektrischen Folgen einer länger dauernden Urea Wirkung, sowie über gewisse Änderungen in der Geschwindigkeit der Repolarisation der Membran nach dem Reiz werden TRAUTWEIN UND GROSSE-SCHULTE in einer gesonderten Mitteilung berichten.

TABELLE VI

EINFLUSS VON 1 *M* UREA AUF MUSKELKONTRAKTUREN

Zimmertemperatur; Kontrakturstoff und Urea sind in Ringer, pH 7.0, gelöst[*]; Belastung bei den Sartoriusmuskeln (110–160 mg) 7–10 g, bei den Rectusmuskeln (350–530 mg) 15–25 g; DAUER der Vorbehandlung ohne oder mit Urea: Sartorien 10 Min, Recti 15 Min; O_2-Durchperlung[**].

Kontrakturstoffe und ihre Konzentrationen	*Muskel*	*Mechanische Arbeit in g × cm/g Muskel und Einwirkungsdauer des Kontrakturstoffes*		
		Ohne Urea	*Mit Urea*	*Nach Auswaschen des Urea*
Avertin[***] 0.5 %	Sartorius	69.3 (5 Min)	0 (5 Min)	67.3 (10 Min)
Avertin[***] 0.5 %	Rectus	66.0 (10 Min)	0 (10 Min)	63.4 (20 Min)
Chloroform gesättigt	Sartorius	38.0 (2 Min)	0 (2 Min)	39.1 (10 Min)
Chloroform gesättigt	Rectus	17.7 (2 Min)	0 (2 Min)	18.5 (10 Min)
Coffein 0.25 %	Sartorius	44.0 (2.5 Min)	0 (5 Min)	43.0 (15 Min)
Coffein 0.3 %	Rectus	67.7 (5 Min)	0 (5 Min)	65.3 (30 Min)
Coffein 0.3 % nach Depolarisierung mit KCl 0.76 %[§]	Rectus	54.1 (3 Min)	0 (3 Min)	57.9 (20 Min)
Acetylcholin 0.1 %	Rectus	25.6 (0.5 Min)	0 (5 Min)	
Cholin 1.0 %	Rectus	14.9 (1 Min)	0 (5 Min)	
KCl 0.76 %	Sartorius	33.1 (0.5 Min)	0 (5 Min)	
KCl 0.76 %	Rectus	22.6 (0.5 Min)	0 (5 Min)	
Nikotin 0.1 %	Sartorius	8.8 (1 Min)	0 (5 Min)	
Nikotin 0.1 %	Rectus	31.6 (2 Min)	0 (5 Min)	
Veratrin 0.2 %	Sartorius	8.6 (0.5 Min)	0 (5 Min)	
Veratrin 0.2 %	Rectus	24.0 (2 Min)	0 (8 Min)	
Monojodacetat 0.1 *M*	Sartorius	7.5 (30 Min)	0 (45 Min)	
Monojodacetamid 0.1 *M*	Sartorius	9.7 (30 Min)	0.2 (60 Min)	
Cetyltrimethylammoniumchlorid 1 %	Sartorius	7.8 (1 Min)	0 (10 Min)	
Saponin 1 %	Rectus	2.9 (2 Min)	0 (10 Min)	

[*] Mit der Ausnahme von KCl, welches ohne und mit Urea in Wasser gelöst ist.

[**] Mit der Ausnahme der Monojodacetat und Monojodacetamid-Versuche.

[***] Tribromäthylalkohol.

[§] Nach[17].

Das Urea macht offensichtlich einen Vorgang unmöglich, der allen Kontraktionen und allen Kontrakturen gemeinsam ist. In Anbetracht der Verschiedenheit der Wege, auf denen Kontraktionen und Kontrakturen ausgelöst werden, dürfte dieses gemeinsame Glied auch im lebenden Muskel das Endglied der Reaktionskette sein, die von der Membran zur Aktivität des kontraktilen Systems führt: die Interaktion zwischen Aktinfilamenten, L-Myosinfilamenten und ATP.

Die Gleichartigkeit der Urea-Wirkung auf den ATP-Mechanismus der Kontraktion im lebenden Muskel und in isolierten kontraktilen Systemen ist bedeutungsvoll. Diese Übereinstimmung ist ein starkes Argument dafür, dass der ATP-Mechanismus der Kontraktion auch ohne Urea im lebenden Muskel und in den kontraktilen Systemen gleich ist.

ANHANG

Die hemmende Wirkung von 1 *M* Urea-Lösung auf die Kontraktion lebender Froschmuskeln trotz annähernd normaler Aktions- und Ruhepotentiale findet ihr mechanisches und elektrisches Analogon in der Unterdrückung der mechanischen Antwort der Muskeln in 2.5–3fach konzentrierter Ringer-Lösung[11–13]. Es lässt sich zusätzlich zeigen, dass auch die Coffein-, Chloroform- und Acetylcholinkontraktur in lebenden Muskeln unterdrückt wird, wenn die Ringer-Konzentration auf das 5fache des normalen Wertes erhöht wird. Auch in diesem Fall werden die Kontrakturen nachgeholt, wenn das störende Agent beseitigt wird, d.h. wenn der Muskel aus der Lösung der Kontraktursubstanz in 5fach Ringer in eine Lösung der Kontraktursubstanz in 1fach Ringer übertragen wird.

Es ist durch SZENT-GYÖRGYI seit langem bekannt, dass die Erhöhung der Salzkonzentration und damit der Ionenstärke in Gegenwart von ATP ebenso Dissoziation des Aktomyosins bewirkt[18] wie die Anwesenheit von Interaktions-Inhibitoren bei niedriger Ionenstärke[2]. Es ist infolgedessen nicht unwahrscheinlich, dass auch der Einfluss hoch konzentierter Ringer-Lösung auf Hemmung der Interaktion von Aktin- und L-Myosin-Filamenten beruht. Die Vorstellung von HILL, die Wirkungen hoher Ringer-Konzentrationen hingen mit dem Zustand des Aktomyosins zusammen[11], gewinnt durch die vorgeschlagene Erklärung eine präzisere Form.

TECHNISCHER TEIL

Versuche mit Modell-Systemen

Die Herstellung des L-Myosins erfolgt nach PORTZEHL, SCHRAMM UND WEBER[19], des Aktins nach MOMMAERTS[20], des natürlichen Aktomyosins nach BÁRÁNY UND BÁRÁNY[21], der Fibrillen nach ULBRECHT UND ULBRECHT[22]. Künstliches Aktomyosin wird von 3 Teilen L-Myosin und 1 Teil F-Aktin hergestellt.

Die Bestimmung der ATPase-Aktivität erfolgt mit 0.1 % Eiweiss, im Histidin-Puffer pH 7.0, in Parallelansätzen. Vor Zugabe des ATP werden die Ansätze 5 Min lang mit Urea oder Acetamid magnetisch gerührt. Die Spaltungzseit dauert 3–10 Min. Es wird darauf geachtet, dass während dieser Zeit nicht mehr als 25 % des zugesetzten ATP gespalten werden. Das freigesetzte anorganische Phosphat wird nach ROCKSTEIN UND HERRON[23] gemessen.

Die Glyzerin-extrahierten Fasern aus dem *M.psoas* werden nach dem Verfahren von A. WEBER[24] hergestellt. Ihr Durchmesser wird mit dem Leitz-Objektiv U.M 3 20 × gemessen. Die Messung der Spannungsentwicklung und des Dehnungswiderstandes erfolgt mit dem von A. WEBER[24] angegebenen Tensiometer. Der Dehnungswiderstand wird durch Dehnung um 10 % der Ausgangslänge und Ablesung nach 2 Min bestimmt. Für die Messung des Dehnungswiderstandes in Anwesenheit von ATP werden Urea und ATP gleichzeitig hinzugefügt. Mit 1 M Urea und $3 \cdot 10^{-3}$ M ATP tritt eine kurze Anfangskontraktion auf (vgl. 2) deshalb erfolgt die Dehnung erst nach Beendigung der Anfangskontraktion. Die Messung ohne ATP erfolgt mit einer frischen Wasser–Glyzerin extrahierten Faser in einem neuen Bad, das nur Urea enthält.

Versuche mit lebenden Muskeln

Die meisten Experimente wurden an Muskelpaaren von *Rana esculenta*, einige auch von *Rana temporaria* zwischen November und Dezember durchgeführt. Die isolierten Muskeln werden an den isotonischen Hebel gespannt und die Ringer-Lösung 15 Min vor dem Anfang und während der Versuche mit O_2 durchperlt. Die Ringer-Lösung enthält: (mM) NaCl, 115.5; KCl, 2.5; $CaCl_2$, 1.8 (siehe 25) und wird vor Gebrauch mit 0.5 mM Natrium Phosphat, pH 7.0, gepuffert. Urea und Acetamid (puriss. Präparate der Fa. Merck) werden in Ringer-Lösung angewendet, ihre Stammlösungen auf pH 7.0 eingestellt. Die Muskeln werden direkt mit einer Kammelektrode durch Kondensatorentladung übermaximal gereizt. (Ein Kondensator von 0.5 μF wird auf 60 V aufgeladen und in 5 Sec Zeitabständen über den Muskel entladen.) Der Reizstrom wird durch eine Kontaktuhr mit Hg-Schalter gesteuert. Die Muskelzuckungen werden an einem Russkymographion registriert. Die Vergrösserung des Hebels ist rund 12fach. Das Volumen des Muskelbades 25 cm^3. Sämtliche Experimente wurden bei Zimmertemperatur durchgeführt. Die Belastung der Muskeln ist in den Legenden und Tabellen angegeben.

Zu den elektrophysiologischen Versuchen werden Sartoriusmuskeln in einer Plexiglaskammer (Volumen 10 cm^3) angewendet. Durch die Kammer zirkuliert eine mit 95 % O_2 und 5 % CO_2 gesättigte Ringer-Lösung bei Zimmertemperatur mit einer Geschwindigkeit von 10 cm^3/Min. Die mechanische Spannung wird mit einem Transducer (RCA 5734) gemessen. Die Ruhe- und Aktionspotentiale einzelner Fasern werden mit Glaskapillarelektroden von LING UND GERARD abgeleitet[26]. Die Verstärkung erfolgt mit Kathodenfolger und Tektronix Oszillograph. Die Elektroden werden mit 3 M KCl gefüllt, ihr Widerstand liegt zwischen 10 und 30 M Ω, ihr Spitzendurchmesser < 0.5 μ. Der Muskel wird im Bad in rechteckigen Spannungsstössen (Grass Stimulator mit Isolation Unit) gereizt. Die Reizelektroden sind zwei bis auf die Spitze isolierte Platindrähte, die an den beiden Muskelrändern liegen.

Bestimmung der Urea-Konzentration in den Muskeln

Die Muskeln werden aus dem ureaenthaltenden Bad herausgenommen, mit Filterpapier abgetupft, auf der analytischen Wage gewogen, mit 4 % Trichloressigsäure geblendert. Der Muskelextrakt wird durch Zentrifugieren isoliert, neutralisiert und ein bestimmter Teil davon in Gegenwart von 0.05 M PO_4, pH 7.0, mit 30 mg Urease (Präparat der Fa. Schuchardt) gemischt. Die Spaltung des Urea erfolgt bei 25°; das freigesetzte NH_3 wird mit Nessler-Reagenz bestimmt. Gleichzeitig wird die Urea-Konzentration im Inkubationsbad gemessen. (Kontrollmuskeln, die mit Urea nicht behandelt sind, besitzen einen NH_3-Gehalt von 0.002 M. Der NH_3-Gehalt der Reagenzien ist praktisch Null.)

Die Berechnung der Urea-Konzentration des Wassers im innern der Muskelfasern

Die Urea Analyse bezieht sich auf 1 g Muskel nach der in Tabelle II angegebenen Zeit der Inkubation im Urea-Bad (Spalte 3 der Tabelle). Für die Berechnung der Urea-Konzentration im freien Wasser des Faserinnern muss bekannt sein (a) der extracelluläre Raum im Muskel, (b) der Anteil des freien Wassers im Muskelgewicht. Der extracelluläre Raum ist nach CONWAY[10] für den normalen Muskel des Frosches zu 0.13 des Muskelgewichtes angenommen. Das freie Wasser ist nach HILL[9] zu 0.77 des Muskelgewichtes angenommen. Beide Werte werden für die Berechnung der unkorrigierten Urea-Konzentrationen verwendet. Für die Berechnung des oberen Grenzwertes im Wasser der Muskelfasern (im Intracellulärraum) wird angenommen, dass die Urea-Konzentration im Intra- und Extracellulärraum des Muskels gleich ist. Der unkorrigierte obere Grenzwert (Spalte 6 der Tabelle II) ist demnach gleich mit der Urea-Konzentration im freien Muskelwasser ($[U]_W$):

$$[U]_W = \frac{[U]_{g\ \text{Muskel}}}{0.77} \tag{1}$$

wobei $[U]_g$ Muskel = Urea-Konzentration in 1 g des Muskels. Für die Berechnung des unkorrigierten unteren Grenzwertes (Spalte 4) wird angenommen, dass die Urea-Konzentration im Extracellulärraum ($[U]_E$) gleich der Konzentration im Bad ($[U]_B$) ist.

Für den Anteil des Extracellulärraumes am freien Muskelwasser (A_E) gilt folgende Beziehung:

$$A_E = \frac{0.13}{0.77} = 0.17 \tag{2}$$

und für den Anteil des Intracellulärraums am Muskelwasser (A_I):

$$A_I = 1 - 0.17 = 0.83 \tag{3}$$

Die Urea-Konzentration im freien Muskelwasser ist gleich:

$$[U]_W = 0.17 \times [U]_E + 0.83 \times [U]_I \tag{4}$$

wobei $[U]_I$ = Urea-Konzentration im Intracellulärraum des Muskels.
Nach Anwendung von $[U]_E = [U]_B$ ergibt sich die Urea-Konzentration im Intracellulärraum oder der unkorrigierte untere Grenzwert:

$$[U]_I = \frac{[U]_W - 0.17 \times [U]_B}{0.83} \tag{5}$$

Für die Berechnung der korrigierten Urea-Konzentration im freien Wasser des Faserinnern wird berücksichtigt, dass das Muskelgewicht durch osmotischen Wasserentzug in Urea-Lösungen abnimmt. Es wird angenommen, dass dieser Wasserverlust nur den Intracellulärraum und nicht den Extracellulärraum betrifft. Der Anteil des freien Muskelwassers am Muskelgewicht (A_W) im Falle eines Gewichtsverlustes (ΔG) wird aus der Formel berechnet:

$$A_W = \frac{77 - \Delta G}{23 + (77 - \Delta G)} \tag{6}$$

(ΔG wird aus Eichkurven entnommen, die ΔG in Abhängigkeit von der Verweildauer in der Urea-Lösung einerseits und dem Anfangsgewicht des Muskels andererseits angeben). Für den Anteil des Extracellulärraums am freien Wasser des osmotischen geschrumpften Muskels ($A_{E,\,0}$) gilt folgende Beziehung:

$$A_{E,0} = \frac{0.13}{A_W} \tag{7}$$

und für den Anteil des Intracellulärraumes am freien Wasser des osmotischen geschrumpften Muskels ($A_{I,\,0}$):

$$A_{I,0} = 1 - A_{E,0} \tag{8}$$

Weiterhin erfolgt die Berechnung der korrigierten Grenzwerte analog der Berechnung der unkorrigierten Grenzwerte. Der korrigierte obere Grenzwert (Spalte 7 der Tabelle II) ist demnach:

$$[U]_{W,0} = \frac{[U]_{g\ \mathrm{osm.\ Muskel}}}{A_W} \tag{9}$$

wobei $[U]_{W,\,0}$ = Urea-Konzentration im freien Wasser des osmotischen geschrumpften Muskels; $[U]_g$ osm. Muskel = Urea-Konzentration in 1 g des osmotischen geschrumpften Muskels; und für A_W ist der Wert einzusetzen der sich aus (6) ergeben hat.

Der korrigierte untere Grenzwert (Spalte 5) ist gleich:

$$[U]_{I,\,0} = \frac{[U]_{W,\,0} - A_{E,\,0} \times [U]_B}{A_{I,\,0}} \tag{10}$$

wobei $[U]_{I,\,0}$ = Urea-Konzentration im Intracellulärraum des osmotischen geschrumpften Muskels; $[U]_B$ = Urea-Konzentration im Muskelbad; und für $[U]_{W,\,0}$, $A_{E,\,0}$ und $A_{I,\,0}$ sind die Werte einzusetzen, die sich aus (9), (7) und (8) ergeben haben.

Bestimmung des Nukleotidgehaltes in den Muskeln

Vor Beginn des Versuches werden die Muskeln gewogen und nach Beendigung des Versuches mit flüssiger Luft zerrieben. Bei 0° wird das Pulver mit 4 %iger $HClO_4$ geblendert, abzentrifugiert, neutralisiert und der Kaliumperchloratniederschlag entfernt.

Wenn die Analyse papierchromatographisch erfolgt, werden die Überstände gefriergetrocknet, in minimalen Mengen von Wasser gelöst, und ein bestimmter Teil davon nach dem Verfahren von KREBS UND HEMS papierchromatographiert[27]. Nach rund 60 Stunden Laufzeit bei Zimmertemperatur ist die Trennung vollständig, die Chromatogramme werden bei 260 mμ photographiert und die Nukleotidflecke nach der Lokalisierung eluiert. Die Identifizierung der Nukleotide erfolgt durch Testsubstanzen einerseits und andererseits spektrophotometrisch bei 250; 260; 280 und 300 mμ. Zur Bestimmung der Nukleotid-Konzentration werden die folgenden molaren Absorptionskoeffizienten benutzt: bei 260 mμ Adenosin-Derivate $14.2 \cdot 10^3$, Inosin-Derivate $7.15 \cdot 10^3$.

Wenn die Analyse säulenchromatographisch erfolgt, werden die Muskelextrakte nach Entfernung des Kaliumperchloratniederschlages alkalisiert (pH 10–11) und auf Dowex X 10 Ionenaustauscher (Durchmesser der Säule 0.5 cm, Höhe 4.0 cm) aufgetragen. Es werden Nukleotidmengen zwischen 2.5–3.0 μMol adsorbiert.

Bei der Analyse der Nukleotidverteilung werden folgende Lösungsmittel angewendet (modifiziert nach[28]): (a) 0.01 M $(NH_4)Cl$, eluiert: Adenosin + Inosin; (b) 0.003 M HCl, eluiert: AMP; (c) 0.006 M HCl + 0.005 M NaCl, eluiert: IMP; (d) 0.006 M HCl + 0.01 M NaCl, eluiert: IMP + ADP; (e) 0.01 M HCl + 0.02 M NaCl, eluiert: ADP; (f) 0.01 M HCl + 0.2 M NaCl, eluiert: ATP.

In einer Stunde laufen 40 ml Lösungsmittel durch die Säule. Die Elution mit demselben Lösungsmittel wird solange fortgesetzt, bis die Absorption bei 260 mμ weniger als 0.02 ist. Die Konzentrationen der verschiedenen Fraktionen werden spektrophotometrisch bestimmt, bei den Wellenlängen 250; 260; 265 mμ. Die Adenosin- und Inosin-Gemische werden nach BENDALL analysiert[29].

Wenn aus den Nukleotiden nur die Menge an ATP bestimmt wird, werden sämtliche anderen Nukleotide aus der Dowex-Säule gleich mit 0.01 M HCl + 0.02 M NaCl entfernt.

ATP-Resynthese von radioaktivem Orthophosphat

Die isolierten M. recti werden 15 Min bei einer Belastung von 10 g in sauerstoffdurchperlte Phosphat–Ringer-Lösung gebracht ($7 \cdot 10^{-3}$ M PO_4, pH 7.0). Dann wird diese Lösung gegen 20 ml einer identisch zusammengesetzten Phosphat–Ringer-Lösung ausgetauscht, die zusätzlich 20 μC ^{32}P/ml und in einem Falle auch 1.0 M Urea enthält. Nach weiteren 15 Min werden die Muskeln aus dem Bad herausgenommen, in 250 ml Phosphat–Ringer-Lösung 2 Min gewaschen um das extracelluläre ^{32}P möglichst zu entfernen. Die Muskeln werden dann mit flüssiger Luft pulverisiert, mit 4 % $HClO_4$ bei 0° geblendert und die Extrakte auf die Dowex-Säule adsorbiert, wie schon früher beschrieben. Die ATP-Fraktion wird ebenfalls mit 0.01 M HCl + 0.2 M NaCl eluiert, und die Konzentration spektrophotometrisch bestimmt. Je 0.5 μMol aus dem isolierten ATP werden auf 50 mg Aktivkohle adsorbiert, und 0.1 ml Triton X zu der Suspension zugegeben. Die Kohle wird in der Zentrifuge (bei 25,000 × g) in Gegenwart von Triton einmal mit 5 % Trichloressigsäure, zweimal mit destilliertem Wasser gewaschen und schliesslich mit 96 %igem Alkohol in kleine Petrischalen quantitativ übergespült. Nach Verdunsten des Alkohols bei 80° wird die Radioaktivität des ATP an der trockenen Kohle bestimmt (nach 30). Es werden 3 mal 3,000 Stösse gezählt und der Leerwert berücksichtigt.

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Wir danken Fräulein R. MÜLLER, Frau E. ARNOLD und Herrn GROSSE-SCHULTE für fleissige und gewissenhafte Mitarbeit, Herrn W. HEINEMANN für die Zusammenstellung des Reizgerätes und für seine Unterstützung bei dieser Arbeit.

ZUSAMMENFASSUNG

1. Urea verwandelt mit steigender Konzentration die Aktomyosin-ATPase von ausgewaschenen Fibrillen und gereinigten Aktomyosingelen in L-Myosin-ATPase. Diese Umwandlung ist abgeschlossen sobald die Urea-Konzentration auf etwa 1 M angestiegen ist. Diese Umwandlung ist reversibel.

2. Bis zur Urea-Konzentration von 1 *M* wird die Aktivität der L-Myosin-ATPase nicht beeinflusst. Die Wirkungslosigkeit von 1 *M* Urea auf die Aktivität der L-Myosin-ATPase ist gemessen an L-Myosingelen und L-Myosinsolen unter Ca und unter Mg.

3. Mit weiterem Anwachsen der Urea-Konzentration bis 2 *M* tritt auch eine geringfügige Inaktivierung der L-Myosin-ATPase ein. Diese scheint nicht oder nicht voll reversibel zu sein.

4. Acetamid wirkt sehr ähnlich wie Urea. Doch ist die Interaktion von Aktin un L-Myosin erst mit 2 *M* Acetamid völlig aufgehoben, während die Schädigung der L-Myosin-ATPase durch Acetamid bereits mit Überschreitung der 1 *M* Konzentration einzusetzen scheint.

5. Auch die mechanische Interaktion der Aktin- und L-Myosin-Filamente wird durch 1 *M* Urea in Gegenwart von ATP aufgehoben. Der ATP-Kontraktion extrahierter Fasern folgt beim Zusatz von 1 *M* Urea eine Erschlaffung. Der Dehnungswiderstand sinkt dabei auf Werte ähnlich Null.

6. Lebende Muskeln antworten in Bädern mit einem Gehalt von 1 *M* Urea nicht mehr auf elektrische Reizung. Diese Hemmung ist voll reversibel. Die analoge Hemmung durch 1 *M* Acetamid ist dagegen unvollständig. 2 *M* Acetamid bewirkt vollständige Hemmung, die aber nur teilweise reversibel ist.

7. Die lebenden Muskeln verlieren in 1 *M* Urea-Bädern nur wenig Wasser, dagegen dringt das Urea schnell bis zum völligen Konzentrationsausgleich in das Faserinnere ein.

8. 1 *M* Urea im Inneren lebender Muskelfasern beeinflusst den ATP-Umsatz des ruhenden lebenden Muskels nicht. Dagegen setzt 1 *M* Urea die ATP-Spaltung stark herab, wenn der lebende Muskel durch Kontrakturstoffe oder durch andere kontrakturerzeugende Faktoren zu erhöhter chemischer und mechanischer Aktivität veranlasst wird.

9. Die chemische und mechanische Hemmung der Muskelaktivität beruht nicht auf einem Urea-Einfluss auf die Membranerregung. Ruhepotential und Aktionspotential bleiben unter 1 *M* Urea praktisch unverändert.

10. Alle untersuchten Arten der Muskelkontraktur (durch 11 verschiedene Kontrakturstoffe) werden durch 1 *M* Urea vollständig und, soweit untersucht, auch reversibel gehemmt. Dieser Erfolg ist davon unabhängig, ob die Kontrakturen unter Depolarisierung polarisierter Membranen stattfinden oder aber auch an depolarisierten Muskeln ausgelöst werden können.

11. Die Gesamtheit der Versuche zeigt, dass die Urea-Wirkung auch im lebenden Muskeln anscheinend an dem letzten gemeinsamen Endglied der Reaktionsketten angreift, die zur Kontraktion oder Kontrakturen führen. Dieses letzte gemeinsame Endglied ist offenbar die Interaktion zwischen Aktinfilamenten, L-Myosinfilamenten und ATP.

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THE EFFECT OF BLOCKING ELECTRICAL ACTIVITY ON THE EFFLUX OF POTASSIUM FROM ELECTROPLAX

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SUMMARY

The effect of various factors which block electrical activity have been tested on the efflux of K from the electroplax of *Electrophorus electricus*.

1. Increase of K concentration from 5 m*M* to 110 m*M* in the Ringer's solution bathing the innervated site of the electroplax, with corresponding decrease in Na concentration, caused an immediate increase of the K efflux. Surprisingly, this increased rate was maintained for a few minutes only and then returned to about the original level. When the K concentration in the outside fluid was raised from 110 to 165 m*M*, no further change of the rate of K efflux was observed.

2. Addition of *d*-tubocurarine in high concentrations (1 mg/ml) did not affect the efflux, thus suggesting that the increased efflux with a high external K concentration must be attributed to an increased flow of K ions across the conducting membrane.

3. When the Ringer K concentration was increased to 110 or 165 m*M*, without corresponding removal of Na, the effects observed with this hypertonic solution were essentially the same.

4. Exposure of the electroplax to either carbamylcholine or to pyridine aldoxime dodeciodide, both known to block electrical activity and to depolarize the conducting membrane, as does high K concentration, affected the rate of efflux of K in essentially the same way as exposure to high K concentration.

5. In contrast, replacement of external Na by either sucrose or choline, in which case activity is blocked without depolarization, did not affect the rate of potassium efflux.

INTRODUCTION

It has previously been shown that during activity, an electroplax from the electric eel, *Electrophorus electricus*, loses 5-8 $\mu\mu$moles of K/cm^2/impulse[1]. This is the same order of magnitude as the K loss from cephalopod and crustacean giant axons[2,3] and suggests the basic similarity of the bioelectric currents propagating the action

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Abbreviation: PAD, pyridine aldoxime dodeciodide.

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potential in membranes of excitable cells from different species. It appeared pertinent to test this conclusion further by studying how various factors which block electrical activity affect the efflux of K. The electroplax preparation possesses certain features discussed by SCHOFFENIELS[4] which make it possible to obtain more information about the mode of action of certain compounds for which other preparations are unsuitable.

The response to direct and indirect stimulation in electroplax can be abolished by replacing the sodium in a saline medium with choline, sucrose or glucose, by increasing the external K concentration and by a number of drugs. By analogy with muscle and nerve, a small amount of Na probably enters the electroplax during stimulation, and its absence in the medium would prevent the inward Na current and the evanescent depolarization during the spike. The block of activity with sufficiently high external K concentrations may be explained by a depolarization of the membrane. The indirect response only is abolished by curare[5,6], which does not depolarize and does not affect the efflux of K from the innervated membranes of resting electroplax[1]. Carbamylcholine, a depolarizing agent, blocks the response to both indirect and direct stimulation although the latter effect is not produced by a direct action upon the conducting membrane but by an action upon the synaptic region[6,7]. A block of both responses is also obtained with lipid soluble quaternary nitrogen compounds such as dodecyl pyridinium chloride and PAD[8].

Several ways are therefore available to block conduction. Since electrical activity of the membrane is accompanied by changes in the efflux of K, it might be expected that a block of activity in various ways would produce changes in efflux characteristic of the mode of blocking which might be of help in the determining of the mechanism of action of drugs. This paper describes the effect of various methods of blocking conduction on the efflux of K from the innervated membranes of electroplax from the organ of Sachs of *Electrophorus electricus* L. The most interesting and unexpected finding is that block with depolarizing agents, like high external K or carbamylcholine or PAD, produces a transient increase in the efflux of K, suggesting that the membrane permeability does not reach a new constant level after depolarization.

METHODS

These were the same as those described in a previous paper[1]. Na free solutions were prepared by replacing Na_2HPO_4 and NaH_2PO_4 with K phosphates, and NaCl with the osmotically equivalent amounts of either sucrose, choline chloride, KCl or K_2SO_4. The pH was 7.2. When chemicals were added to the Ringer's solution, the pH was re-adjusted, if necessary, to 7.2 with either 0.1 *N* NaOH or 0.1 *N* HCl.

RESULTS

Effects of high external potassium concentration

Reversibility of the block of conduction: A single electroplax separating two pools of Ringer's solution in the apparatus developed by SCHOFFENIELS[4,6,9] was stimulated directly and the amplitude of the spike was about 60 mV (Fig. 1). To find the effect of an elevated K concentration on the amplitude of the spike, the fluid washing the innervated membrane was replaced with Ringer's solution in which sodium had been replaced with potassium to give concentrations of 25, 45 and 80 m*M* K. Fig. 1 shows that the height of the spike was reduced by the raised K concentrations. It fell only about 5 mV with 25 m*M* K and on washing again with Ringer's solution, the spike immediately returned to its initial level. With 45 m*M* K the height of the spike was reduced by 50 % and on washing with Ringer's solution, it again recovered completely within about 3 min. 80 m*M* K caused a block of conduction and on washing with Ringer's solution afterwards, the height of the spike slowly increased until after 20 min it was 85 % of its initial level. Both the direct and indirect responses were also recovered after their abolition with 165 m*M* K, irrespective of whether NaCl was replaced by KCl (Fig. 2) or K_2SO_4 (Fig. 3). The recovery in the height of the spike was slower (40 min) and not so complete (75 %) in the presence of sulfate compared with the 85 % recovery in 20 min in the presence of chloride. Similar results were obtained when both sides of the electroplax were washed with high K Ringer's solution and show that the block of conduction was reversed when the electroplax was washed afterwards with Ringer's solution.

The efflux of K into isotonic solutions: To find the effect of the block of conduction by K on the efflux of K across the innervated membrane, electroplax previously soaked for 2–3 h in oxygenated labeled Ringer's solution were mounted in the apparatus with a pool of ^{42}K labeled Ringer's solution on the non-innervated side and a stream of inactive Ringer's fluid on the innervated side. The washing fluid was counted for ^{42}K until a constant rate was obtained; then it was replaced by a high K solution. The K concentrations chosen, of 110 and 165 m*M*, were obtained by a replacement of sodium of the saline with potassium. Fig. 4 shows that washing the innervated membrane with Ringer's solution containing 110 m*M* K caused an immediate increase in the loss of ^{42}K from the electroplax. Surprisingly, the high rate of loss of ^{42}K was not maintained for more than about 10 min, for after that time it fell slowly to the initial level. (The spike disappeared within about 30 sec after exposure to the high K.) To test whether a further transient increase in the loss of ^{42}K would occur on raising the external K concentration to 165 m*M*, the washing fluid was altered to contain 165 m*M* K and Fig. 4 shows that no further change in

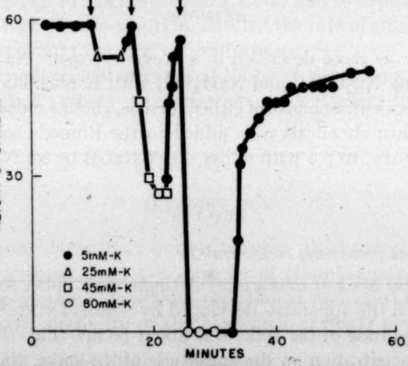

Fig. 1. Decrease in the height of the direct spike on increasing the potassium concentration in the Ringer's solution bathing the innervated membrane.

the efflux of K occurred. In 2 experiments the electroplax was bathed in high K Ringer's solution and subsequently washed with 5 m*M* K Ringer's solution until the electrical activity returned. The efflux of K was allowed to fall to the initial level, after the transient increase, before the start of washing with Ringer's solution. No further change occurred as the electrical activity was recovered after about 30 min washing.

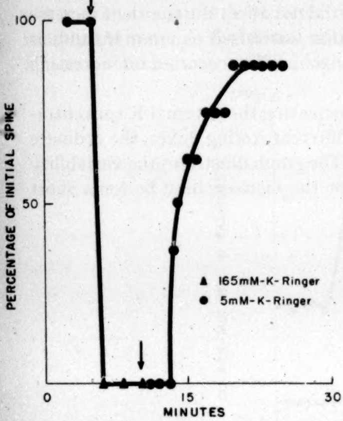

Fig. 2. Reversibility of the block of activity after bathing the innervated membrane with 165 m*M* K Ringer's solution.

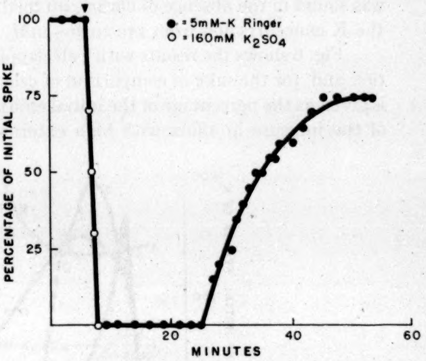

Fig. 3. The reversibility of the effect of 160 m*M* K_2SO_4 solution on the direct spike.

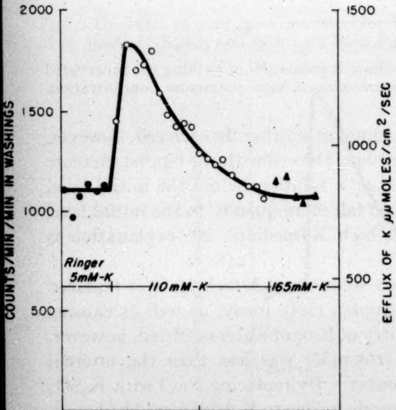

Fig. 4. The efflux of potassium from the innervated membrane of an electroplax on bathing with Ringer's solution in which sodium had been partially or completely replaced with potassium.

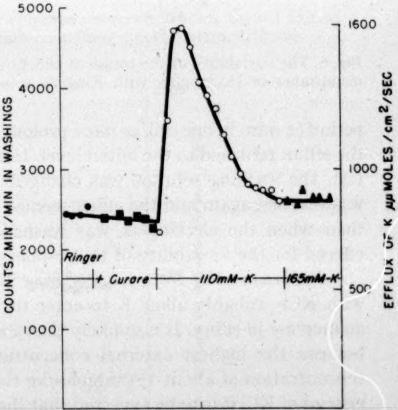

Fig. 5. The efflux of potassium from the innervated membrane of an electroplax whilst bathing it in the presence of curare and of high potassium concentrations.

Fig. 5 shows that the addition of *d*-tubocurarine (1 mg/ml) to the Ringer's solution bathing the innervated membrane had no significant effect on the efflux of K, although the indirect response was blocked, showing that nearly all the resting efflux is probably through the conducting membrane. This is not surprising since the area of the post-synaptic membrane at the synaptic junctions, in spite of their great number, forms only a small fraction, about 3–6 %, of the conducting membrane[10]. The previous abolition of the indirect spike with curare did not affect the transient increase in the loss of ^{42}K when the external K concentration was raised to 110 m*M*, and, as was found in the absence of curare, no further change in efflux occurred on increasing the K concentration from 110 to 165 m*M*.

Fig. 6 shows the results with 3 electroplax of increasing the external K concentration and, for the sake of comparison of cells with different resting fluxes, the ordinate is given as the percentage of the initial efflux of K. The graph illustrates the variability of the increase in efflux with high external K, for the increase may be for a short period (2 min) in one cell, or more prolonged (12 min) in another. In each cell, however, the efflux returned to the initial level. In the curve of the cell with the biggest increase (□), the washing solution was changed to Ringer's solution before the initial level was reached again and the efflux seemed then to fall more quickly to the initial level than when the electroplax was washed with high K medium. No explanation is offered for the variability of the response.

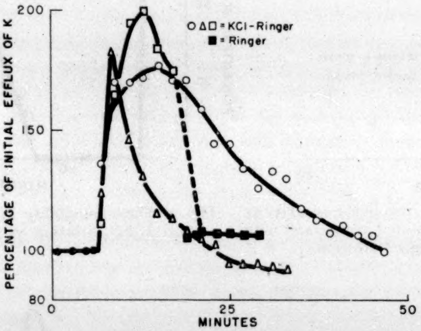

Fig. 6. The variability of the transient effect on the efflux of potassium of bathing the innervated membranes of electroplax with Ringer's solution containing a high potassium concentration.

Replacement of NaCl with K_2SO_4: Ringer's solutions in which NaCl is replaced with KCl probably allow K to enter the electroplax more freely, as well as causing an increase in efflux. It is unlikely that a net entry of K or of water occurred, however, because the highest external concentration (165 m*M*) was less than the internal concentration of about 175 mmoles/kg tissue water[11]. By replacing NaCl with K_2SO_4 instead of KCl it is to be expected that the same effects due to K itself would be found. Fig. 7 shows that this is so, for when the NaCl in Ringer's solution was replaced with 0.083 *M* K_2SO_4, the same transient increase in the efflux of K occurred as when NaCl was replaced with KCl.

Addition of KCl to Ringer's solution: To test the effect on the efflux of K of maintaining the Na concentration in the Ringer's solution at 165 m*M* and of increasing the K concentration, hypertonic solutions were prepared by adding solid KCl to Ringer's solution to raise the K concentration to 110 or 165 m*M* K solutions. Fig. 8 shows a 110% increase in the loss of ^{42}K from the electroplax when the direct spike was blocked with 110 m*M* K in a hypertonic medium and also the subsequent fall to the initial level. Washing with 165 m*M* K solution caused no further change in efflux from the initial level, after the pre-treatment with 110 m*M* K. This result is exactly the same as that found with isotonic high K solutions in which NaCl of the Ringer's solution was replaced with KCl.

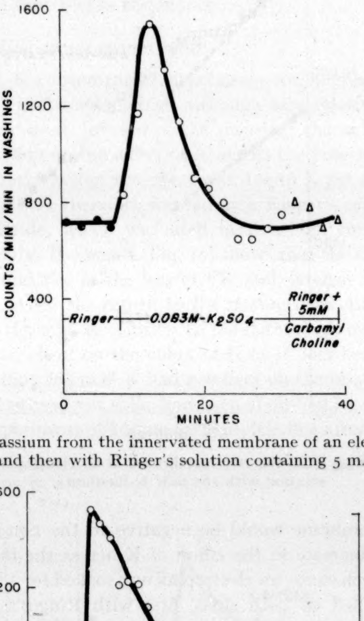

Fig. 7. The efflux of potassium from the innervated membrane of an electroplax bathed first with 0.083 *M* K_2SO_4 and then with Ringer's solution containing 5 m*M* carbamylcholine.

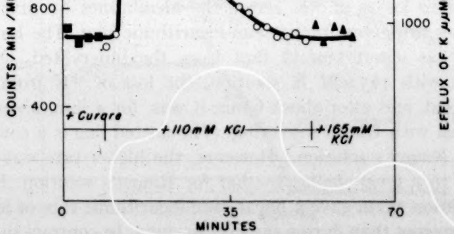

Fig. 8. The efflux of potassium from the innervated membrane of an electroplax bathed with Ringer's solution to which had been added solid KCl to give high potassium concentration.

Washing both membranes of the electroplax: The results already described in which high K Ringer's solution was added only to the innervated side could possibly be artifactual due to a net potential difference (p.d.) across the cell caused by a depolarization of only the innervated membrane which might cause a flow of current carried by K. (With high K on the innervated side only, a net p.d. would be expected across the cell since there would still be a p.d. across the non-innervated membrane and the innervated membrane would be negative to the non-innervated.) To test whether the transient increase in the efflux of K across the innervated membrane might be due to some such cause, an electroplax was soaked for 3 h in labeled Ringer's solution and then washed on both sides, first with Ringer's solution, next with isotonic 165 m*M* K solution and finally again with Ringer's solution. The washings were counted for ^{42}K and the results are shown in Fig. 9. Initially, the rates of loss corresponded to losses of ^{42}K across the membranes by first order processes since straight lines are given in the semi-logarithmic plot. The loss from the non-innervated side was about tenfold that from the innervated. On replacing the Ringer's solution with 165 m*M* K solution, the loss of ^{42}K from the innervated membrane increased, and after about 6 min it was, for 4 min, about twice the rate it would have been with Ringer's solution. (The dotted line is a continuation of the linear slope with Ringer's solution.) However, the higher rate was not maintained and the rate fell to a level similar to that for Ringer's solution. Further washing with Ringer's solution again gave a linear semi-logarithmic rate of loss although the slope was a little steeper than during the first washing. In contrast to the marked rise and fall in the loss of ^{42}K found on the innervated side, no striking change was seen

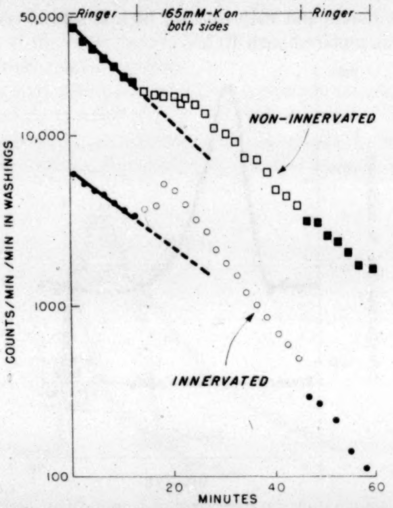

Fig. 9. The efflux of potassium from the two membranes of an electroplax on replacing Ringer's solution with 165 m*M* K-medium.

in the washings from the non-innervated side. In 3 experiments made in this way the same effect was observed on the innervated side. In one experiment, the loss from the non-innervated membrane also showed a rise (middle section of upper curve in Fig. 9) although this was not so pronounced. The results therefore show that the same rise and fall in the loss of ^{42}K occurred from the innervated membrane when both sides were washed with 165 m*M* K as when only the innervated side was washed. The effect found when washing the innervated side alone is therefore unlikely to be an artifact and may be considered to be the typical way in which the efflux of potassium changes when the membrane is exposed to a high potassium concentration (110 or 165 m*M*) that blocks conduction.

Effect of carbamylcholine on potassium efflux

A high external K concentration depolarizes the membrane[12–14] and the same result is produced by carbamylcholine[6], although at a relatively slow rate with the concentrations usually used. In view of the transient character of the effect of high external K concentration on the efflux of K across the innervated membrane, it was clearly desirable to find whether the effect was due to K *per se*, or to its depolarizing action. An electroplax was therefore washed with Ringer's solution containing 5 m*M* carbamylcholine chloride, which was used in this high concentration in order to depolarize fairly quickly. The result (Fig. 10) shows that the drug blocked conduction and also caused a rapid rise in the loss of ^{42}K and, later, a slow fall approximately to the starting level. After the return to the resting level, the washing solution was changed to isotonic 165 m*M* K solution to see if the high external K concentration would still produce an effect on the efflux of K as it does before treatment with the drug. Fig. 10 shows that 165 m*M* K had no effect on the loss of ^{42}K across the innervated membrane once the direct spike had been abolished by carbamylcholine.

The converse experiment of adding carbamylcholine after high potassium showed

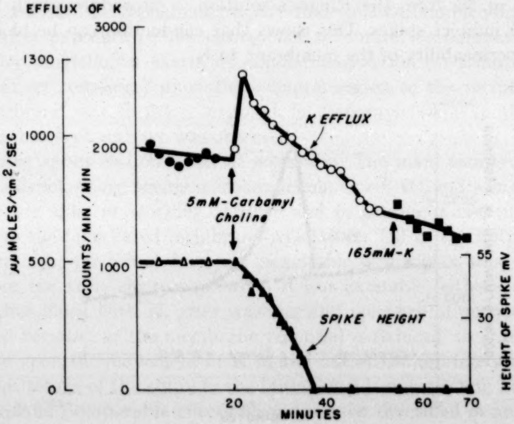

Fig. 10. The efflux of potassium and the height of the direct spike in the presence of 5 m*M* carbamylcholine and of 165 m*M* K in the Ringer's solution bathing the innervated membrane.

that no further change in efflux occurred with carbamylcholine once the spike had been abolished with 165 mM K solution. Since the peculiar effect on the efflux of K is the same with carbamylcholine as that with high external K concentrations, and since the response is abolished by pre-treatment with the other agent, the results suggest that the same reaction in the process of membrane depolarization is the cause of the phenomenon.

Effect of 2-PAD on potassium efflux

A drug that abolishes the direct spike even after the indirect spike has been abolished by curare in high concentrations, is 2-PAD, which therefore affects the conducting membrane directly[8]. Carbamylcholine affects the direct spike by way of the nerve synapses only[6] and was therefore used in the absence of curare. The innervated membrane of an electroplax was exposed to 1 mg/ml of curare, to abolish the indirect spike, and then to 400 μg/ml of 2-PAD. The effect of PAD on the efflux of K (Fig. 11) is the same as the effect of carbamylcholine and of a high external K concentration in producing a temporary rise in the loss of ^{42}K which then fell in about 10 min to the original level. The direct spike was abolished within 2 min after exposure of the innervated membrane to Ringer's solution containing PAD.

Block of conduction by removal of external sodium

The results have shown that, when activity was abolished by high external K, by carbamylcholine or by PAD, the efflux of K from the innervated membrane increased rapidly by about 70–100% and then fell to the original level. The common feature of these blocking agents is membrane depolarization. To find the effect of block of activity without depolarization, the efflux of K has been measured from electroplax whose conduction had been blocked by replacing external Na ions with choline or sucrose. This procedure does not affect the resting membrane potential[12]. The results showed that the efflux of K from the resting cell was not affected by a removal of Na from the Ringer's solution, a procedure which blocked both the direct and indirect spikes. This shows that conduction can be blocked without changing the permeability of the membrane to K.

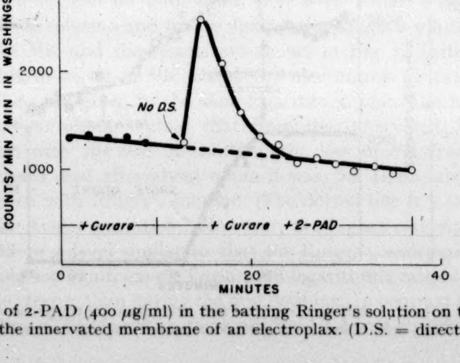

Fig. 11. The effect of 2-PAD (400 μg/ml) in the bathing Ringer's solution on the efflux potassium from the innervated membrane of an electroplax. (D.S. = direct spike).

DISCUSSION

The block and recovery of activity

The experiments described in this paper were designed to find out whether different methods of blocking electrical activity in electroplax from the organ of Sachs of *Electrophorus electricus* affected the efflux of K in characteristic ways. Block of activity has been obtained in two ways, viz. by removal of Na from the medium and by the action of depolarizing agents like K and drugs. Replacement of Na in Ringer's solution with choline or sucrose does not affect the resting potential[12]; presumably the driving force on K movements is essentially the same and it is not surprising that the efflux of K from the resting, yet unexcitable, electroplax was also unchanged. It was also shown[12] that the block obtained in sucrose medium was reversed on washing with Ringer's solution but not after immersion in choline medium; the reason for this difference remains to be explained.

Block of activity was obtained by exposing either the innervated membrane alone or both the innervated and non-innervated membranes to Ringer's solution in which NaCl had been partially (about 50 %) or completely replaced with KCl or K_2SO_4. The block was reversed on washing with the usual Ringer's solution containing 5 m*M* K. Loss of activity occurred within 1 min, in contrast to the longer time (about 30 min) needed to restore activity.

A similar long period of washing is needed to restore the resting potential of frog muscles after they have been immersed in high K solutions[15], and a slow repolarization of the membrane is the likely explanation for the slow recovery of excitability in electroplax.

The block of the direct response obtained with the lipid soluble quaternary nitrogen compound, 2-PAD, after the abolition of the indirect spike by the addition of *d*-tubocurarine, confirms SCHOFFENIELS *et al.*[8] and is probably due to the combination of 2-PAD with, and inactivation of, constituents of the membrane necessary for activity[10]. 2-PAD is a depolarizing agent[8] and so is carbamylcholine, but whilst 2-PAD acts after exposure of the electroplax to curare, carbamylcholine is then ineffective[6]. Carbamylcholine exerts its depolarizing action (and block of both the indirect and direct responses) upon the synaptic region of the membrane; the innervated membrane was therefore exposed to carbamylcholine in the absence of curare and the block of activity was observed.

Depolarizing agents and the efflux of potassium: The main results of this paper show that the depolarizing agents, carbamylcholine, 2-PAD and a high external K concentration are alike in blocking activity and in causing a rise in the efflux of potassium from the innervated membrane which later fell to the initial level. After the evanescent rise, the efflux from an inexcitable electroplax was thus identical with that from the same electroplax when it was excitable, either before block of activity or, after block with K, after washing and recovery of activity. The rise is to be expected because, as the membrane potential is reduced, so is the restraint of the membrane upon the movement of K in and out of the cell likely to be reduced. The subsequent return of the efflux to the initial level is unexpected, however, and is difficult to explain. Comparable effects have not been described in any other tissue and, indeed, the results with crab nerve showed that the efflux increased five-fold and remained constant at the high level when the external K concentration was also increased five-fold[16]. In *Sepia* nerve axons poisoned with 2,4-dinitrophenol there was also a very rough proportionality between the external concentration and the efflux of potassium over the range from 10 to 207 m*M* K (see ref. 17). This work showed that depolarization increased the conductance of the membrane and that K ions carry the current; the experiments did not indicate transitory effects like those described here.

It is worth examining the evidence against the view that the effect is artifactual since this possibility is the most obvious and least demanding. First the result could be spurious if depolarization allowed a net loss of K from the electroplax which might account for the extra radioactivity in the washings. However, since it may be calculated that the total radioactivity collected above the initial level represented only about 5% of the intracellular K, the return of the efflux to the initial level cannot be explained by a loss of cell K. This result also rules out the possibility that the non-innervated membrane might become the limitation to the movement of tracer from the pool of Ringer's solution on the non-innervated side of the electroplax to the washing solution on the other side. A net loss of K from the electroplax is unlikely when bathing with 110 or 165 m*M* K solutions, but this calculation shows that it also was unlikely to occur as a result of the action of carbamylcholine or 2-PAD when the external K concentration was 5 m*M*.

Secondly, the fall in efflux might be due to a decrease in the specific activity of the intracellular K upon which the efflux is directly dependent. A fall in efflux due to a lowering of intracellular specific activity should be related to the external K concentration, which determines the amount of K that can enter the cell, since the internal specific activity would fall to a greater extent as more K entered. This possibility can be excluded because the efflux returned to the same initial levels during exposure of the electroplax to carbamylcholine, 2-PAD and high K concentrations, when different amounts of K would be expected to enter the cell because of the different external concentrations (5, 110 and 165 m*M*). The fact that the efflux did not fall to lower levels with 110 and 165 m*M* external K than with 5 m*M* shows that entry of external K could not have lowered the internal specific activity in a way that would explain the fall to the same initial levels. These two considerations argue that the effect of depolarizing agents on the efflux of K is not an artifact but the typical response of the innervated membrane.

Although at the moment there is no explanation for the phenomenon of the transient change in K efflux in the electroplax there are several hypotheses that can be tested and the isolated electroplax is interesting material for further study of the nature of the system controlling potassium permeability.

ACKNOWLEDGEMENTS

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AN IMPROVED ISOLATED SINGLE ELECTROPLAX PREPARATION

II. COMPOUNDS ACTING ON THE CONDUCTING MEMBRANE

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SUMMARY

Effects on the electrical activity of an isolated single electroplax preparation were studied with several compounds which act at the conducting membrane as well as at the synapses. Two types of compounds were tested:

1. Tertiary amines. Some of them are potent inhibitors of acetylcholinesterase and of the acetylcholine receptor (physostigmine and the tertiary analogue of Prostigmine). Others are mainly receptor inhibitors: procaine, tetracaine, and dibucaine ("local anesthetics") and atropine. All these compounds block electrical activity without depolarization. Their reaction with the receptor protein has been recently demonstrated in solution.

2. Lipid soluble quaternary ammonium ions: pyridine aldoxime dodeciodide, noracetylcholine, and norcholine. These compounds act on the acetylcholine system, but the affinities to the various protein members seem to be influenced by concentration and structure; depending on a variety of factors block of response may be observed with or without depolarization. Atropine, norACh and norCh affected the indirect response in about 0.1 the concentration required to affect the directly evoked spike. The other compounds affected the response to both types of stimulation about equally. All compounds tested affected the directly evoked spike in approximately the same concentration in the presence as in the absence of curare. Physostigmine and T.P. are twice as potent blockers of electrical activity at pH 6 as at pH 9. At pH 6 the molecules, in solution, are present in their cationic, at pH 9 in their uncharged form.

INTRODUCTION

In a preceding paper[1], using the improved preparation developed by SCHOFFENIELS[2], we have described the effects of acetylcholine and related quaternary nitrogen structures which act primarily at the synaptic regions of the electroplax. In the present paper

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Abbreviations: TP, tertiary analogue of prostigmine; PAD, pyridine-2-aldoxime dodecyliodide; ACh, acetylcholine; M.A.C., mininal active concentrations; DIB, dibucaine; AT, atropine; PHYS, physostigmine.

* BT-519.

** BT-526

we will examine the effects upon electrical activity of compounds which penetrate structural barriers more readily since they are lipid soluble and therefore act upon the acetylcholine system of the conducting as well as of the synaptic membrane of the electroplax. They are mostly tertiary amines which combine with the acetylcholine receptor, but apparently do not readily produce a change of configuration due to the absence of the tetrahedral quaternary nitrogen group. They block the response to direct and indirect stimulation without depolarization. These compounds are, therefore, called receptor inhibitors[3]. Some of them are potent cholinesterase inhibitors. The directly evoked spike is blocked in about the same concentration in the presence of curare as in its absence. To determine the relative potency of the charged *versus* uncharged amine, physostigmine and T.P. were tested at pH 6 and 9.

Some experiments were also performed with lipid soluble quaternary ammonium ions in which a dodecyl group substitutes one methyl group on the nitrogen. The compounds studied are PAD, β-acetoxyethyl dimethyl dodecyl ammonium iodide (noracetylcholine dodeciodide-norACh 12), and β-hydroxyethyldimethyl dodecyl ammonium iodide (norcholine dodeciodide-norCh). These lipid soluble analogues of acetylcholine block the direct response of the cell in the presence of curare like tertiary amines, but in contrast to the latter they are able to depolarize[4].

The methods used for isolating a single electroplax are the same as described in preceding papers[1, 2].

RESULTS

Table I lists the compounds tested and the M.A.C., *i.e.*, that which causes at least a 50% decrease in the height of the action potential within 30 min or less (usually between 10 and 30 min). The reasons for using this index of activity were discussed previously[1].

Procaine, tetracaine and dibucaine, usually referred to as local anesthetics, are receptor inhibitors[3]. Their potencies upon frog sciatic nerve fibers have been compared and correlated with their lipid solubilities[5–10]. Since they are tertiary ammonium derivatives it will depend upon the pH as to whether the nitrogen atom has a positive charge

TABLE I

MINIMAL ACTIVE CONCENTRATIONS* OF COMPOUNDS WHICH ACT AT THE CONDUCTING MEMBRANE AS WELL AS AT THE SYNAPSE

The first three compounds affected the direct (d) and indirectly (i) elicited responses about equally.

Compounds	*No. of Expts.*	*M.A.C. μg/ml*	*Time (min)*
Procaine	4	125–250	10–20
Tetracaine	3	10–20	10–15
Dibucaine	4	5–10	10–20
PAD	6	5–25	10–30
NorACh 12	3	(d) 50–100	5–20
	3	(i) 5	10–20
NorCh	3	(d) 25–100	20–30
	3	(i) 5	10–20
Atropine	4	(d) 200–2000	20–30
	4	(i) 200	20–30

* Concentration required to decrease spike height by 50% within 30 min or less (see last column).

or not. All drugs listed in Table I were dissolved in Ringer's solution of pH 7.0–7.5.

Fig. 1 shows an example of the results obtained with dibucaine, a much more potent local anesthetic than procaine. Fig. 2 shows the usual response obtained with

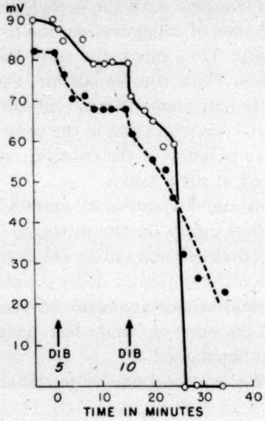

Fig. 1. Effect of DIB on the electrical activity of an isolated single electroplax of *Electrophorus electricus*. The compound was added to the pool bathing the innervated membrane. The response (in mV) to direct ●---● and indirect ○—○ stimulation as recorded by an oscilloscope using extracellular electrodes. The concentrations in μg/ml are indicated by the figures below DIB.

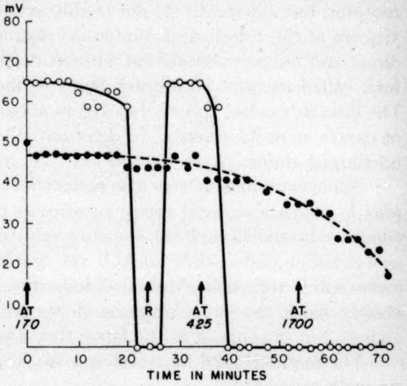

Fig. 2. Effect of atropine (AT) on the electrical activity of an isolated single electroplax. Recordings and symbols as in Fig. 1. The concentrations in μg/ml are indicated by the figures below AT. At R the preparation was washed with Ringer's solution.

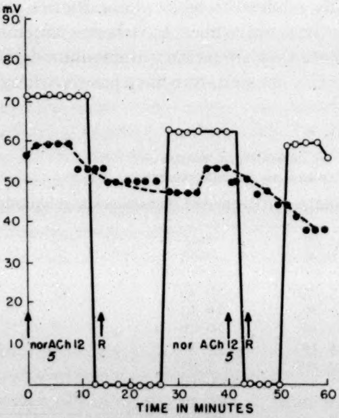

Fig. 3. Reversibility of noracetylcholine dodeciodide action on the electrical activity of an isolated single electroplax. Recordings and symbols as in Fig. 1. The concentrations (μg/ml) are indicated by the figures below norACh 12. At R the preparation was washed with Ringer's solution.

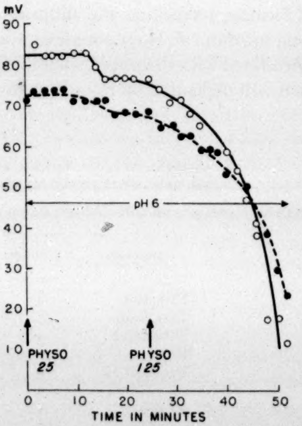

Fig. 4. Effect of PHYS at pH 6 on the electrical activity of the isolated single electroplax.

atropine; *i.e.*, a block of the indirectly elicited spike in a lower concentration than the direct. Complete reversal of block by atropine of indirect stimulation is readily obtained and at least a 50 % reversal of the directly elicited spike. The effects of 5 to 25 μg/ml PAD were usually irreversible although in one case we obtained a 50 % reversal of block of the indirect response. Block of the indirectly stimulated spike by norACh 12 (5 μg/ml) was at least 50 % reversible as shown in Fig. 3. No return of electrical activity occurred after block by 50–100 μg/ml norACh 12 nor any effective concentration of norCh.

Since the acetylcholine receptor is thought to possess an anionic site[3], it was of interest to compare the effect upon electrical activity of the charged *versus* the uncharged form of a tertiary amine. Physostigmine and T.P. were therefore tested at pH 6 and 9. Tris (hydroxymethyl)aminomethane in 1.5 m*M* concentration, was used as the buffering agent in these experiments instead of phosphate buffer in order to avoid precipitation at alkaline pH. The buffer did not alter the electroplax action potential. Both of the compounds were somewhat weaker at the basic pH as shown in Table II and Figs. 4 and 5. The mean ± the standard error for the M.A.C. of physostigmine at pH 6 was 280 ± 76 μg/ml which was significantly less ($P < 0.01$) than the value at pH 9 (890 ± 253 μg/ml). Under identical conditions neostigmine did not vary in potency with a change of pH from 6 to 9.

TABLE II

EFFECT OF pH UPON THE ACTION OF PHYSOSTIGMINE AND THE TERTIARY ANALOGUE OF PROSTIGMINE

Compound	*pH*	*No. of Expts.*	*M.A.C. μg/ml*	*Time (min)*
Physostigmine	5.5–6.5	5	100–400	10–25
	8.5–9.5	5	200–1300	10–25
Tertiary Analogue of Neostigmine	5.5–6.5	5	1000	20–30
	8.5–9.5	4	2000	15–30

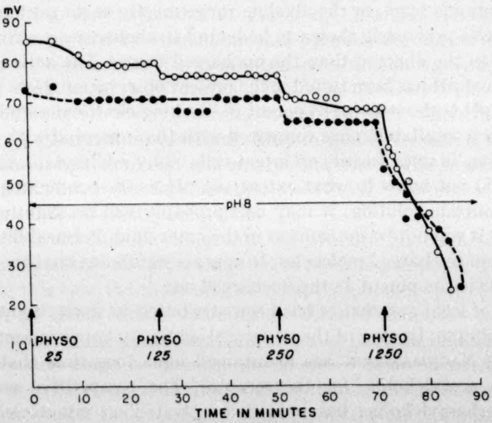

Fig. 5. Effect of physostigmine at pH 8 on the electrical activity of the isolated single electroplax.

All compounds listed in Tables I and II were applied to an electroplax following block of the indirectly elicited spike by 50 μg/ml curare. The compounds to be tested were in all cases combined with 50 μg/ml curare to ensure that the synaptic receptors remained blocked. All were found about as effective in blocking the directly evoked response, following block of indirect response by curare, as they were in the absence of curare, indicating they can act directly upon the conducting membrane. Physostigmine was about 0.5 as potent at pH 8 as at pH 6 in affecting the directly evoked response, which is similar to what was observed in the absence of curare.

DISCUSSION

Tertiary amines

Two types were tested: (a) compounds known to have strong inhibitory action on cholinesterase (physostigmine and the tertiary analogue of Prostigmine), (b) compounds referred to as local anesthetics, presumed to act mainly on the receptor. Physostigmine is also a receptor inhibitor, and blocks electrical activity of the electroplax before the enzyme concentration reaches a critically low level, as was shown by NACHMANSOHN *et al.*[11]. The view that eserine reacts with the receptor protein has recently been confirmed by studies of EHRENPREIS on the acetylcholine receptor protein in solution. He tested by equilibrium dialysis the binding of a variety of compounds to the receptor protein[12,13]. A striking parallelism became apparent between the strength of binding to the protein in solution and the effectiveness on the electrical activity of the isolated single electroplax. Whereas eserine is a potent receptor inhibitor, the affinity of T.P. towards the receptor protein has not yet been evaluated. T.P. is about 100 times weaker as an enzyme inhibitor than its quaternary analogue.

The existence and importance of an anionic site in the active surface of acetylcholinesterase and of the receptor protein has been demonstrated in many ways and discussed elsewhere[3]. Dimethylaminoethyl acetate was shown to be more potent in blocking electrical activity at pH 6 than at pH 9 (see ref. 1). In the acid range it is present in its cationic form, in the alkaline range mostly as an uncharged molecule. Physostigmine was previously shown to be bound to cholinesterase in solution about 16 times better in the charged than the uncharged form[14]. The action on the intact cell as a function of pH has been tested in the present observation. Both physostigmine and T.P. are at pH 6 about twice as potent in blocking electrical activity as at pH 9. This may appear a small difference compared with that observed with the enzyme in solution. However, in experiments on intact cells many additional factors determine the effect. We do not know to what extent the pH in the conducting membrane is altered by the outside solution; it may and probably will be slightly affected, but almost certainly it will not be the same as in the outer fluid. Permeability barriers will favor the action of uncharged molecules. It appears significant that the charged form is nevertheless twice as potent as the uncharged one.

The action of local anesthetics has been attributed to some "tightening" of the conducting membrane. In view of the structural similarity between acetylcholine and local anesthetics NACHMANSOHN has maintained for a long time that they act by competing with acetylcholine for the receptor. The competitive action between procaine and carbamylcholine has been demonstrated on intact electroplax[11]. In recent observations (S. EHRENPREIS AND M. G. KELLOCK, unpublished experiments) it was found that procaine, tetracaine and dibucaine are bound to the acetylcholine receptor protein in solution. The latter two are much more strongly bound than procaine. It appears pertinent that here again a striking parallelism exists with the effect of the compounds on the intact electroplax. Moreover, tetracaine specifically precipitates receptor protein in contrast to curare which reacts with many other macro-molecules. These developments form a new and strong support for NACHMANSOHN's view on the role of acetylcholine in conduction. The data demonstrate the essentiality of the acetylcholine receptor protein. Considered in combination with the evidence that cholinesterase activity is inseparable from electrical activity, the new facts make it extremely difficult to offer an alternative interpretation.

Atropine blocked the indirect response usually in a lower concentration than required to affect the directly elicited spike. This agrees with the pharmacological observation that atropine in low doses acts primarily at certain synaptic regions. Curare also blocks the indirect response in a much lower concentration than the direct[1], although the concentration difference is much greater than with atropine. This is probably due to the difference in penetration, since when atropine is quaternized and made lipid insoluble by a polymethylene chain, the resulting bis atropinium compound is a potent curarizing agent[15]. Bis atropinium compounds because of their lipid insolubility would not penetrate as well as atropine, and because of their large size might not depolarize[1].

Lipid soluble quaternary ammonium ions

NorACh and PAD, in 10^{-3} *M* concentration, have been found to depolarize the electroplax[4]. In the present observations electrical activity was affected by PAD in about 50 times and norACh in about 10 times lower concentrations than previously reported. However, the criterion used, the minimum active concentration to reduce the activity 50 %, is not quite comparable. Whether the membrane was depolarized has not been ascertained since only extracellular electrodes were used.

Relatively small concentrations of norACh and norCh (5 μg/ml) block the response to indirect but not to direct stimulation. This is the first dissociation observed with these lipid soluble quaternary ammonium ions between the action on synaptic and that on conducting membranes. The concentration acting on the synaptic junction is about 5 times that found with acetylcholine. The concentrations acting on the conducting membrane are about 50 times higher than the concentration of acetylcholine acting on the synaptic junction[1]. This 10 fold difference is relatively small compared to that for lipid insoluble quaternary compounds. The presence of structural barriers surrounding the conducting membrane, whatever their properties, may readily account for some differences in permittivity.

The low concentrations blocking only the synaptic junctions do not seem to depolarize. Depolarization would be expected to block the response to direct stimulation, because in this case the thousands of synaptic junctions per electroplax would produce a short circuiting of the conducting membrane[16]. Thus, low concentrations of these two quaternary ammonium ions seem to act as receptor inhibitors. It is, therefore, interesting that there is virtually no difference between norACh and norCh. Choline is known to be about 2000 times weaker than acetylcholine as a depolarizing agent. It is even weaker than tetramethyl and trimethylethyl ammonium ions. For some reason the oxygen atom may possibly prevent the binding with the receptor. It is remarkable that when a dodecyl group is substituted for a methyl, the difference between choline and acetylcholine virtually disappears.

PAD, in 5–25 μg/ml, blocks the response to both direct and indirect stimulation simultaneously, suggesting that PAD even in low concentrations may cause depolarization. Such an assumption is supported by the observations of DETTBARN on Ranvier nodes of single sciatic nerve fibers[17].

Although evidence has accumulated that the lipid soluble quaternary ammonium ions in relatively low concentrations, act on conducting membranes by affecting the acetylcholine system, their affinity to the various protein members may differ, dependent on concentrations and on special structural features. They may release acetylcholine from the storage protein, they may act on the receptor either as activators or inhibitors, and finally they may inhibit acetylcholinesterase. Acetylcholinesterase prepared from electric tissue of *Electrophorus* was found to be inhibited *in vitro* about 60 % by 10^{-6} *M* PAD and 70 % by 10^{-4} *M* norCh (HANNA GREENBERG, unpublished experiments). The pertinent fact of the present as well as of previous observations on the action of these lipid soluble quaternary ammonium ions is the evidence that they act on the synaptic and on the conducting membrane in a way similar to other compounds which specifically affect the acetylcholine system.

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ACETYLHEXOSAMINE COMPOUNDS ENZYMICALLY RELEASED FROM *MICROCOCCUS LYSODEIKTICUS* CELL WALLS

III. THE STRUCTURE OF DI- AND TETRA-SACCHARIDES RELEASED FROM CELL WALLS BY LYSOZYME AND *STREPTOMYCES* F$_1$ ENZYME*

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SUMMARY

Two compounds, both of which have been isolated from cell walls of *Micrococcus lysodeikticus* digested with lysozyme or *Streptomyces* F$_1$ enzyme, have been identified as di- and tetra-saccharides. The reducing groups of the di- and tetra-saccharides and those of the high-molecular weight, non-dialysable compounds belong to muramic acid. β-glucosidase yields free N-acetylglucosamine and N-acetylmuramic acid from the di- and tetra-saccharides. The proposed structure for the di-saccharide liberated by lysozyme and F$_1$ is: 6-O-β-N-acetylglucosaminyl-N-acetylmuramic acid. The tetra-saccharides isolated from lysozyme and F$_1$ digests appear to be identical. The structure proposed for the tetra-saccharide isolated from lysozyme digested walls is: O-β-N-acetylglucosaminyl-(1→6)-O-β-N-acetylmuraminyl-(1→4)-O-β-N-acetylglucosaminyl-(1→6)-β-N-acetylmuramic acid.

Both lysozyme and *Streptomyces* F$_1$ enzyme degrade di- and tetra-chitobiose, indicating their β(1→4) N-acetyl hexosaminidase activity.

INTRODUCTION

It has been known for some time that the enzyme lysozyme, brings about a liberation of N-acetylamino sugar compounds from the "mucopolysaccharide" substrates isolated from bacterial cells[1,2]. Subsequent investigations with isolated cell walls as "substrate" established that lysozyme liberated N-acetylamino sugars but none of the amino acids present in the cell-wall mucopeptides[3]. SALTON[3] presented evidence for the release of a di-saccharide of N-acetyl-glucosamine and N-acetylmuramic acid as the simplest product of digestion of the substrate with lysozyme. BERGER AND WEISER[4] found that lysozyme degraded chitin [aβ(1 → 4)N-acetylglucosamine polymer]. Thus it became apparent that lysozyme belonged to the N-acetylhexosaminidase group of enzymes,

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Abbreviations: AG, N-acetylglucosamine; AMA, N-acetylmuramic acid; DNP, dinitrophenyl-; FDNB, 1, fluoro-2,4-dinitrobenzene; NDF, non-dialysable fraction.

[*] A preliminary account of this work appeared[11].

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and it seemed likely from the results of BERGER AND WEISER[4], that the enzyme is specific for the β-glycosidic bond. It will be recalled that MEYER, PALMER, THOMPSON AND KHORAZO[5] had concluded a long time ago that the linkages broken by lysozyme were definitely not α-glycosidic bonds. BRUMFITT, WARDLAW AND PARK[6] have also advanced the hypothesis that lysozyme is a $\beta(1 \longrightarrow 4)$ N-acetyl hexosaminidase and from a differential test for the reaction of N-acetylamino sugars suggested that the reducing group liberated by the enzyme was that of AMA.

With the development of a more sensitive reaction for detecting N-acetylamino sugars[7] additional products in the dialysable fractions of lysozyme and Streptomyces enzyme F_1, digests of *M. lysodeikticus* walls have been detected[7,8]. This paper presents the details of attempts to establish the structure of a compound corresponding to the di-saccharide reported earlier[3,7,8] and an oligo-saccharide[8]. Independent confirmation of the existence of the disaccharide has been reported by PERKINS[9,10] and the structure he has recently suggested is in complete agreement with that proposed by SALTON AND GHUYSEN[11].

EXPERIMENTAL

Unless otherwise specified, the compounds used throughout these studies were those isolated as described by GHUYSEN AND SALTON[8], from lysozyme and Streptomyces F_1 enzyme digests of *Micrococcus lysodeikticus* (NCTC 2665) cell walls.

Preparation of ^{14}C compounds

Cell walls containing ^{14}C in glucosamine and muramic acid were prepared from *M. lysodeikticus* grown with aeration at 30° on a medium containing 0.4 % Difco casamino acids, 0.05 % Difco yeast extract and [^{14}C] fructose, or [^{14}C] glucose. The organism was cultivated on a medium of the same composition with non-radioactive fructose or glucose (0.8 %) prior to washing (with the casamino acids–yeast extract mixture) and replacement in the radio-active medium. The walls were isolated and radioautographs of the hydrolysed walls (6 *N* HCl, 16 h, 105°) showed that labeling of the wall compounds was almost exclusively confined to glucosamine, muramic acid and glucose with only very faint traces in wall amino acids such as glutamate and lysine.

Di- and oligo-saccharides were isolated from [^{14}C] wall material after digestion with lysozyme (Armour Laboratory, crystalline egg-white lysozyme) for 24 h at 37°, pH 7, 0.1 *M* ammonium acetate buffer. The dialysable fraction (material diffusable on dialysis for 48 h at room temperature) was then placed as a band on Whatman paper No. 1 and using *n*-butanol–acetic acid–water (3:1:1, v/v) the compounds suspected of being di- and tetra-saccharides separated cleanly from one another, and from the slower moving, peptide-amino sugar complexes. On the elution of the two compounds and re-running on paper with the same solvent systems, they were found to be homogeneous—each compound gave a single spot on radioautographs.

Reaction with $NaBH_4$

Reduction of amino sugar compounds with $NaBH_4$ has been successfully used by DAVIDSON AND MEYER[12,13] in their studies on the structure of mucopolysaccharides. We have investigated the reaction of our compounds with $NaBH_4$ as well as treating the NDF of lysozyme-digested walls and the untreated walls with this reagent.

The oligo-saccharides, the walls and NDFs were treated with an excess of $NaBH_4$ (Metal Hydrides, Inc., Beverly, Mass.) at 0° for 2 h. The reaction mixtures were then warmed to 20° and held at that temperature for periods varying from 30 min to 90 min, prior to decomposition of the reagent with acetic acid in the cold and adding sufficient acid to bring the pH to 5. Walls were washed free of the reagents by centrifugation and the NDF was dialysed against several changes of distilled water.

The di- and oligo-saccharide were reduced with $NaBH_4$ in the following media: (a) distilled H_2O; (b) 0.1 *M* borate buffer, pH 8.0; (c) 0.05 *M* borate buffer, pH 9.2. The reaction was allowed to proceed at 0° and samples were taken at intervals and tested for loss of ability to give a MORGAN AND ELSON reaction on paper (Whatman, No. 1) using the conditions described by SALTON[7]. Loss of MORGAN AND ELSON reaction occurred most rapidly in unbuffered solution. Weak positive reactions for N-acetylamino sugars were obtained even after 2 h reduction with $NaBH_4$ in borate buffer at both pH 8.0 and pH 9.2, indicating incomplete reaction under these conditions. In one experiment, reduction was prolonged to 12 h at 0° and a small amount of residual material was still detectable with the sensitive spray reagent. Reaction products were generally held for 90 min at 20° prior to destruction of the reagent. [^{14}C] compounds were treated in an identical manner.

Examination of the products of reduction with $NaBH_4$

All preparations reduced with $NaBH_4$ were hydrolysed with 2 *N* or with 6 *N* HCl (for 3 or 16 h respectively) at 105°, the HCl removed by drying *in vacuo* over H_2SO_4 and NaOH pellets. The hydrolysates were examined by two-dimensional paper chromatography using pyridine–water (4:1, v/v) in one direction and *n*-butanol–acetic acid–water (3:1:1, v/v) in the second direction. Paper chromatograms were sprayed with ninhydrin, the $AgNO_3$ reagents of TREVELYAN, *et al.*[14], and the Schiff's reagents as described by BUCHANAN *et al.*[15].

With the exception of the intact, untreated cell-wall preparation, all other reduced fractions showed the appearance of a new compound on examination of the hydrolysates by paper chromatography. This substance reacted more weakly with ninhydrin than the parent amino sugars, it reacted with $AgNO_3$ and gave a white spot with the Schiff's spray reagent[15]. The new compound was well separated from both glucosamine and muramic acid on paper chromatograms and did not correspond to the position of a sample of glucosaminitol prepared by reduction of AG with $NaBH_4$ (the reduced AG was de-ionized and then de-acetylated by hydrolysis). That the new substance was the reduction product of muramic acid was supported by its separation in the neutral fraction when the hydrolysis products of the reduced fractions were submitted to electrophoresis at pH 6.4 (pyridine–acetic acid buffer[16]), 10 V/cm. This evidence indicated the presence of both amino and carboxyl groups. The substances therefore possessed all the properties of the amino sugar hexitol of muramic acid. A sample of muramic acid isolated by paper chromatographic separation of hydrolysed *M. lysodeikticus* wall, upon reduction with $NaBH_4$ gave as a major product, a compound of similar behaviour on electrophoresis and on paper chromatography as that detected in the reduced substances under investigation. The compound will thus be referred to subsequently as "muramicitol" (the amino sugar hexitol of muramic acid).

Neither muramicitol nor glucosaminitol was detected in hydrolysates of walls treated with $NaBH_4$. The products detected in the oligo-saccharides, NDF and cell walls submitted to reduction, are summarized in Table I.

A determination of the ratios of the products would give confirmatory evidence

TABLE I

PRODUCTS OF THE REDUCTION OF WALLS, NDF AND OLIGOSACCHARIDES WITH $NaBH_4$

Preparation	*Enzyme used*	*Products of acid hydrolysis*			*Other compounds*
		Glucosamine	*Muramic acid*	*Muramicitol**	
Walls	None	+	+	—	Amino acids, glucose[3]
NDF	Lysozyme, F_1	+	+	+	Amino acids, glucose[3]
Di-saccharide	Lysozyme, F_1	+	Trace	+	±**
Tetra-saccharide	Lysozyme, F_1	+	+	+	±**

* Muramicitol — $R_{F\text{muramic acid}} = 0.75$ } *n*-butabol–acetic acid–water (3:1:1)
Glucosaminitol — $R_{F\text{muramic acid}} = 0.35$ }

** Small amounts of what is believed to be de-acetylated disaccharide which has resisted hydrolysis; $R_{F\text{muramic acid}} = 0.22$.

as to the structure of the compounds. The ratio of muramicitol to glucosamine in the di-saccharides (from both lysozyme and F_1 enzyme digests) was determined by conversion of the hydrolysis products of the reduced compound to the DNP derivatives by reaction with FDNB under the conditions successfully applied to the determination of amino sugars in blood group substances by LESKOWITZ AND KABAT[17]. The DNP compounds were separated on paper chromatograms (Whatman 3MM paper saturated with phthalate buffer and irrigated in one direction with *tert.*-amyl alcohol as described by BLACKBURN AND LOWTHER[18] and using the 1.5 *M* phosphate buffer of LEVY[19] for the second dimension). The DNP compounds were eluted in 1 % w/v $NaHCO_3$ and the extinctions measured at 360 mμ. A ratio of 1:0.8 was obtained for the proportion of DNP-glucosamine to DNP-muramicitol. The DNP-glucosamine and DNP-muramicitol could also be separated by electrophoresis on 3MM paper (10 V/cm, 2–3 h, pH 6.4). The products from the oligosaccharide could not be determined by this method, as the conditions used did not separate the DNP derivatives of muramic acid and muramicitol.

The ratios of muramicitol to glucosamine and muramic acid were therefore determined with the use of ^{14}C-labeled compounds. After reduction, the compounds were hydrolysed (2 *N* HCl, 3 h, 105°) and the three substances glucosamine, muramic acid and muramicitol were separated on Whatman paper No. 1 with *n*-butanol–acetic acid–water (3:1:1, v/v), the compounds were eluted, concentrated and then the radioactivity in the fractions determined. The results for the lysozyme di- and

TABLE II

RATIOS OF ACID HYDROLYSIS PRODUCTS IN $NaBH_4$-TREATED [^{14}C] DI- AND TETRA-SACCHARIDES ISOLATED FROM LYSOZYME-DIGESTED [^{14}C] WALL

	Di-saccharide		*Tetra-saccharide*	
	Counts/min	*Ratio**	*Counts/min*	*Ratio**
Glucosamine	261	1.6	302	2.5
Muramicitol	162	1	115	1
Muramic acid	70	0.4	121	1.1

* Relative to muramicitol.

tetra-saccharide are presented in Table II, adding further confirmation as to the identity of one compound as a di-saccharide and supporting the impression gained from paper chromatography that the other oligo-saccharide is a tetra-saccharide.

The experiments with the [^{14}C] compounds confirm the presence of some muramic acid after reduction, thus indicating incomplete reaction. This would in part explain the lower yields of muramicitol in the reduced di- and tetra-saccharides. Recent experiments performed by one of us (M.R.J.S.) has revealed the presence of an additional product in the reduced preparations which may also account for the low values for muramicitol. Thus it is likely that some degradation of the muramic acid occurs under conditions leading to reduction and such compounds are readily detectable when radioautographs of the hydrolysed products are examined (SALTON, unpublished observations).

Oxidation with $NaIO_4$

The linkages between the N-acetylamino sugars in the di- and tetra-saccharides were investigated by attempting to determine the liberation and possible origin of formaldehyde on oxidation with $NaIO_4$. As the isolated di- and tetra-saccharides were not 100 % pure[8], ambiguous results on periodate consumption and formaldehyde liberation were anticipated. This was confirmed with some preliminary experiments using the spectrophotometric method of determining periodate consumption and determining the H·CHO colorimetrically[21]. To avoid the difficulties of determing the origin of any H·CHO formed, [^{14}C] di- and tetra-saccharides (from lysozyme and F_1 digests) were used.

Oxidation with $NaIO_4$ was performed at pH 7.5, at 2° for 30 h under conditions giving maximum H·CHO liberation from AG[22] and from blood group substances[23]. At the end of oxidation, carrier H·CHO was added, followed by an excess of 5,5-dimethyl-1,3 cyclohexandione (dimedon) and the ^{14}C contents of the insoluble H·CHO-dimedon compounds were determined. A preliminary experiment in which a large excess (5 mg) of carrier H·CHO was added showed a small amount of radioactivity in the dimedon precipitates. With [^{14}C] di-saccharide (3,260 counts/10 min) and [^{14}C] tetra-saccharide (3,060 counts/10 min) [both from wall isolated from cells grown on [^{14}C] fructose and digested with lysozyme] the radioactivity in the H·CHO-dimedon precipitates was 7 counts/10 min and 19 counts/10 min respectively; a control sample of [^{14}C] di-saccharide treated in an identical manner, but without $NaIO_4$, showed a similar level of activity (18 counts/10 min) in the precipitates. [^{14}C] glucose oxidized under these conditions gave 75 % of the theoretical yield of [^{14}C] formaldehyde which on adjustment for absorption of the radioactivity (determined by mixing [^{14}C] glucose with an equivalent weight of non-radioactive H·CHO-dimedon) indicated a 97 % liberation of formaldehyde.

[^{14}C] di- and tetra-saccharides isolated from walls derived from *M. lysodeikticus* grown on [^{14}C] glucose were dissolved in 0.3 ml H_2O and 0.3 ml $NaHCO_3$ buffer containing $NaIO_4$ was added and oxidation allowed to proceed for 30 h at 2° in the dark[22]. [^{14}C] glucose was treated in the same manner, and controls of di- and tetra-saccharides together with a [^{14}C] glucose control were treated under the same conditions but in the absence of $NaIO_4$. At the end of oxidation the following reagents were added to each tube: 3 drops *N* HCl; 1 ml 0.1 *N* Na_2AsO_3; 0.14 ml formaldehyde solution containing 500 μg H·CHO; 1 ml *M* sodium acetate; 1 ml 5 % w/v dimedon in ethanol. The contents of the tubes were mixed thoroughly and allowed to stand at room temperature for complete formation of the H·CHO–dimedon complexes. The H·CHO–dimedon precipitates were collected on membrane filters, washed several times with ice-cold distilled water, dried and the ^{14}C contents determined. The controls showed no activity in the precipitates. The results for the $NaIO_4$-treated compounds are summarized in Table III.

TABLE III

DETERMINATION OF THE [^{14}C] FORMALDEHYDE LIBERATED ON PERIODATE OXIDATION OF [^{14}C] GLUCOSE AND [^{14}C] DI- AND TETRA-SACCHARIDES

^{14}C Compound	*Initial radio-activity*	*[^{14}C] in H.CHO-dimedon*	*Theoretical ^{14}C when C_6 available for oxidation*	
	Counts/min	*Counts/min*	*Counts/min*	*% theory*
Di-saccharide	6,080	0	405*	0
Tetra-saccharide	3,670	0	122	0
Glucose	11,900	1,930	1,980	97.5

* Values expected for a 1 → 4 linkage with C_6 of the reducing sugar free to react with $NaIO_4$.

The action of β-glucosidase on di- and tetra-saccharides

The nature of the linkages between AG and AMA has been investigated by examining the products formed after incubation with β-glucosidase (Mann Research Laboratories Inc., salt-free, almond β-glucosidase). Approximately 400 μg of each compound (lysozyme and F_1 di- and tetra-saccharides in 0.1 ml 0.1 *M* sodium acetate buffer, pH 5.0, were added to 0.1 ml of the enzyme solution (500 μg enzyme in 0.1 ml 0.1 *M* sodium acetate buffer). The mixtures were incubated at 30° for 48 h, together with control solutions of the four compounds (no enzyme added). The reaction mixtures were examined by paper chromatography (Whatman No. 1, *n*-butanol–acetic acid–water; 3:1:1, v/v) using the N-acetylamino sugar spray described by SALTON[7]. All four compounds incubated with the enzyme gave substances separating in an identical manner to free AG and free AMA. Only the original di- and tetra-saccharides were detectable in the absence of β-glucosidase. In addition to the free acetylamino sugar constituents, residual di- and tetra-saccharides were detectable on the chromatograms, indicating incomplete cleavage of the compounds by β-glucosidase. Elution of the free AG and AMA from the paper chromatograms as described by SALTON[7] and estimation of the colours at 585 mμ gave ratios of AG to AMA of 1:1.3 for both lysozyme and F_1 disaccharides (the greater colour of the AMA is probably due to the presence of borate in the spray reagent, an effect used by PARK[24] for a differential test for AMA). That the compounds believed to be free AG and AMA were indeed these substances was further substantiated by their behaviour on fingerprinting (electrophoresis followed by chromatography) under the conditions used by SALTON[7]. Electrophoresis at pH 6.4 indicated the presence of a free carboxyl group in the compound corresponding to AMA.

Incubation of the NDF from lysozyme digested walls of *M. lysodeikticus* with β-glucosidase under the conditions used above, also liberated free AG and AMA from the 10,000–20,000 molecular weight[3] mucopeptides. Untreated cell walls incubated similarly did not show the presence of free acetylamino sugars.

The action of lysozyme and Streptomyces F_1 enzyme on di- and tetra-chitobiose

In view of the further degradation of the tetra-saccharides liberated from walls by lysozyme and F_1 upon further incubation of the isolated compounds with the enzymes, it was of interest to determine whether these enzymes were capable of degrading N-acetylglucosaminides of known structure. Di- and tetra-chitobiose, kindly provided by Professor S. ROSEMAN, were incubated with lysozyme and F_1 enzyme using the following conditions: 500 μg each compound in 0.05 ml H_2O were added to enzyme (25 μg) dissolved in 0.05 ml 0.2 M ammonium acetate buffer, pH 7, and the mixtures were incubated for 48 h at 37°.

Examination of the products on Whatman paper No. 1 irrigated with *n*-butanol–acetic acid–water (3:1:1) and spraying for acetylaminosugars[7], revealed the presence of two compounds in the enzyme-treated di-chitobiose (original material and a substance separating very closely to AG) and three compounds in the tetra-chitobiose incubation mixture (original tetra-chitobiose, a compound with an R_F identical to that of di-chitobiose and a third substance separating as AG). Whether the substance separating closely to AG, is in fact AG or chitobiose (4-O-β-N-acetylglucosaminyl-N-acetylglucosamine) has not been established. These results do show, however, that both enzymes possess $\beta(1 \rightarrow 4)$-N-acetylglucosaminidase activity.

DISCUSSION

The results of our investigations and those of PERKINS[9,10] have established the simplest product of the action of lysozyme and Streptomyces F_1 enzyme as a di-saccharide of AG and AMA. The isolation of a tetra-saccharide, the structure of which we would like to propose as: O-β-N-acetylglucosaminyl-$(1 \rightarrow 6)$-O-β-N-acetylmuraminyl-$(1 \rightarrow 4)$-O-β-N-acetylglucosaminyl-$(1 \rightarrow 6)$-β-N-acetylmuramic acid provides experimental verification of earlier suggestions by BRUMFITT, WARDLAW AND PARK[6] and BERGER AND WEISER[4] as to the nature of the linkages split by lysozyme. We can conclude that the enzymes lysozyme and Streptomyces F_1 break similar glycosidic bonds, although it is likely, as GHUYSEN[25] has pointed out, that there are differences in affinities for the substrate. Further structural evidence for the existence of the $(1 \rightarrow 4)$ linkage between the two di-saccharide residues of the tetra-saccharide is being sought, although the important discovery that both lysozyme and F_1 degrade the isolated tetra-saccharides to the di-saccharide[25] together with the above results of their action on known $\beta(1 \rightarrow 4)$-N-acetylglucosaminides and the action of β-glucosidase strongly support the proposed structure.

It is of interest to note that both the di- and tetra-chitobioses can be detected on paper with the spray reagent described by SALTON[7], despite the fact that substitution in the C_4 position usually abolishes or greatly reduces the MORGAN AND ELSON reaction as shown by JEANLOZ AND TRÉMEGE[26]. Thus detection of the products of digestion of walls with the MORGAN AND ELSON reagents did not preclude the possibility of $1 \rightarrow 4$ linkages. The results of PERKINS[10] and our evidence clearly establish $1 \rightarrow 6$ linkages in the di-saccharide. It may be argued that the absence of [^{14}C] formaldehyde from the $NaIO_4$-treated compounds is not conclusive evidence of a $1 \rightarrow 6$ linkage. However, although experiments with reduced [^{14}C] compounds do yield some radio-activity in the H·CHO–dimedon complexes, the amounts are not compatible with a 1 → 4 structure and it seems likely that they originate from degradation products formed on treatment with $NaBH_4$ (SALTON, unpublished data).

The type of structure constituting the cell walls of certain lysozyme-sensitive bacteria was originally proposed by SALTON[27] from the results of the investigation of the products of digestion of the walls by lysozyme. It was thus suggested that the walls possessed a structure "in which peptides and peptide-amino sugar complexes may be glycosidically linked through terminal amino sugars to other amino sugar and sugar residues of polysaccharide components"[27]. With the characterization of muramic acid[28,29] and the muramic acid-nucleotide-peptide[30] a more detailed structure for the wall was proposed by BRUMFITT, WARDLAW AND PARK[6]. The results of PERKINS[10] together with the above investigations and those of GHUYSEN AND SALTON[8] and GHUYSEN[25] provide experimental evidence for the existence of an AG–AMA backbone in the wall and suggest the following type of structure for the wall of *M. lysodeikticus* (Fig. 1).

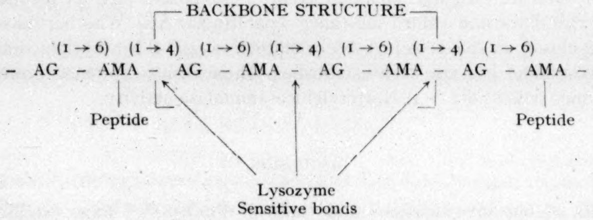

Fig. 1.

Little can be said about the structure of the peptide part of the wall mucopeptide. Although the amino acid ratios in some of the diffusable compounds isolated by GHUYSEN AND SALTON[8] are identical with those for the whole cell wall, the analysis of the products of partial acid hydrolysis of the wall of *M. lysodeikticus* by PERKINS AND ROGERS[31] shows a more complicated variety of peptides. With the isolation of a compound containing glucosamine, muramic acid and glycine in approximately equimolar proportions[31], it is conceivable that some glycine residues may be present as substituent groups and not in a peptide structure. The detection of substantial amounts of "C-terminal" glycine residues in the walls of *M. lysodeikticus* submitted to hydrazinolysis would be compatible with this view (SALTON, unpublished results).

NOTE ADDED IN PROOF

Although specific activities of ^{14}C compounds were not determined, it is evident from the results of RICHMOND AND PERKINS[32] that under our growth conditions the casamino acids would depress the labelling of the lactyl residue of muramic acid to give ratios observed in Table II.

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STUDIES OF THE BACTERIAL CELL WALL

VII. MONOSACCHARIDE CONSTITUENTS OF THE WALLS OF GRAM-NEGATIVE BACTERIA

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SUMMARY

The DISCHE reaction and paper chromatography have been used in detecting and determining the distribution of heptoses and other monosaccharide constituents in walls of Gram-negative bacteria and in wall-fractions obtained after removal of capsular polysaccharide, and after extraction with diethylene glycol, trichloroacetic acid and treatment with lysozyme and 1 % acetic acid. Heptoses were found in the lysozyme-insoluble fractions of walls containing these sugars. Diethylene glycol and trichloroacetic acid gave polysaccharides in soluble and insoluble fractions whereas treatment of walls with 1 % acetic acid releases most of the polysaccharide. It is concluded that the "surface" antigenic polysaccharides are part of the lipopolysaccharide-protein portion of the wall.

Unknown compounds which do not appear to be 3,6-dideoxy sugars have been detected in the walls of *Alcaligenes faecalis* and *Rhodospirillum rubrum*.

INTRODUCTION

The chemical constitution of the walls of Gram-negative bacteria is more complex than that of walls of Gram-positive organisms[1,2]. Mucocomplex substances (mucopeptides and mucopolysaccharides) together with the teichoic acids[3] may account for the entire cell-wall structure of many Gram-positive organisms, whereas the mucopeptide moiety of the walls of Gram-negative bacteria may constitute only a small percentage (10–20 %) of the weight of the cell wall[4,5]. Thus, the major groups of substances in the walls of Gram-negative bacteria include proteins, lipids, polysaccharides and mucocomplex components.

The monosaccharide constituents of the "surface antigens" of Smooth strains of Gram-negative bacteria[6] have been detected in isolated cell walls[1]. Dideoxy sugars[7,8] and heptoses[9,10] have been isolated from Smooth and Rough antigenic, bacterial polysaccharides respectively. WEIDEL[11] isolated a heptose from the wall of *Escherichia coli* B. Although the characteristic sugars of the antigenic polysaccharides have been detected in the wall preparations, there has been no direct experimental evidence establishing the polysaccharides as integral parts of the cell-wall structure, with the

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Abbreviations: DEG, diethylene glycol; TCA, trichloracetic acid.

exception of the lipo-polysaccharide studied by WEIDEL *et al.*[4,11,12]. In the absence of any experimental information as to the anatomical status of the Smooth antigenic polysaccharide complexes, WILKINSON[13] and SALTON[14] have referred to these as "micro-capsular" constituents. The studies presented in this paper represent an attempt to obtain some information about the anatomical origin of the monosaccharide constituents in isolated cell-wall preparations obtained from Gram-negative bacteria.

MATERIALS AND METHODS

Organisms and isolation of cell walls

Cell walls were isolated from the following organisms: *Alcaligenes faecalis* (NCTC 8764); *Bacterium cadaveris* (NCTC 6578); *Chlorobium thiosulphatophilum*; *Chromobacterium kilense* (NCTC 4619); *Chromobacterium prodigiosum* (NCTC 2847); *Escherichia alkalescens* (NCTC 4338); *Escherichia dispar* (4169); *Klebsiella aerogenes* (NCTC 8172, and strains A3, A3(Sl), A3(O) kindly provided by Dr. J. F. WILKINSON), *Klebsiella cloacae* (NCTC 5920), Organism LC 1 (ELSDEN *et al.*[15]), *Photobacterium albensis* and *Photobacterium fischeri* kindly provided by Dr. R. SPENCER; *Proteus vulgaris*; Pseudomonas sp. kindly provided by Dr. A. D. BROWN; *Rhodospirillum rubrum*; *Salmonella gallinarum*; *Spirillum serpens*. Washed suspensions of cells harvested from suitable media were subjected to the wall isolation procedures previously described[16]. All of the cell-wall fractions were digested with trypsin prior to final washing and freeze-drying.

DISCHE *reaction for detection of certain monosaccharides*

Cell-wall preparations were examined by the DISCHE reaction[17] under conditions used for the detection of heptoses. 1–2 mg of dried wall or cell-wall fractions were placed in test tubes and 1 ml water added prior to the addition of the H_2SO_4 with cooling in an ice bath. After the addition of the H_2SO_4, the tubes were transferred to a water-bath at room temperature and then placed in a vigorously boiling water-bath and heated for 3 min. The tubes were cooled to room temperature and 0.1 ml 3% w/v cysteine–HCl (or 0.1 ml H_2O for control tubes) was added and the contents mixed and the spectra were determined after standing for 24 h at room temperature.

Detection of dideoxy sugars

Compounds separating on paper chromatograms in the vicinity of dideoxy sugars were eluted from papers and subjected to the reaction for 3,6-dideoxy-aldohexoses described by FROMME, LÜDERITZ, STIERLIN AND WESTPHAL[18].

Paper chromatography of monosaccharide constituents

Cell-wall preparations were hydrolysed with 0.5 *N* H_2SO_4 for 8–16 h, or with 2 *N* H_2SO_4 for 2 h at 100° in sealed ampoules. Hydrolysates were neutralized with $Ba(OH)_2$ and after removal of the $BaSO_4$, the preparations were either dried and dissolved in pyridine for placing on the chromatogram paper or they were de-ionized with a mixed resin bed [Amberlite resins IR-120 (H) and IR-4B (OH)] concentrated and dissolved in distilled water to give solutions containing about 5% sugars. Separation of monosaccharides was performed on Whatman paper No. 1, with *n*-butanol–pyridine–water (6:4:3, v/v) or *n*-butanol–acetic acid–water (6:1:2, v/v) as the solvent systems. Paper chromatograms were sprayed with aniline phthalate[19] or with the $AgNO_3$ reagent described by TREVELYAN *et al.*[20]. Specific detection of glucose was performed with the "Glucostat" reagent (Worthington Biochemical Corporation) as described by SALTON[21].

RESULTS

Distribution of monosaccharides in wall fractions from capsulated, slime-producing and non-encapsulated strains of Kl. aerogenes

To determine whether the monosaccharides of the capsular and slime polysaccharides are detectable in the isolated cell-wall fractions, three strains of *Kl. aerogenes* previously investigated by WILKINSON, DUGUID AND EDMUNDS[22] were selected. The *Kl. aerogenes* A3 strain possesses a capsular polysaccharide which may be extracted with hot water[23]. The absorption spectra of DISCHE reaction products of wall fractions of the A3 strain before and after removal of the capsular polysaccharide are compared in Fig. 1 with that of the extracted polysaccharide material. The monosaccharide constituents detectable in the cell-wall fractions of the capsulated (A3), slime-producing (A3 Sl) and the non-encapsulated A3(O) strains of *Kl. aerogenes* are summarized in Table I, together with the results obtained with a serologically related strain[22], *Kl. cloacae*, and an additional strain of *Kl. aerogenes* (NCTC 8172).

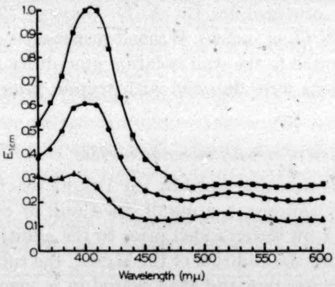

Fig. 1. ●—●, spectra of products of DISCHE reaction on *Kl. aerogenes* A3 untreated walls; ▲—▲, walls extracted with hot water; ■—■, hot-water extractable capsular polysaccharide. $E_{1\ cm}$ for 0.45 mg each fraction.

Detection of heptoses by the DISCHE *reaction*

Cell walls have been subjected to the DISCHE reaction using the conditions favourable for the detection of heptoses[17]. From these preliminary tests with the DISCHE reaction, heptoses have been suspected in the cell walls of a number of organisms used in these studies. The heptoses give an absorption peak in the range 505–510 mμ (see ref. 17) and a typical example of results obtained with bacterial walls is presented in Fig. 2, showing the absorption spectra of *Proteus vulgaris* walls subjected to the DISCHE reaction. It will be seen from Fig. 2 (curve 2) that the walls treated with the H_2SO_4 alone (no cysteine added) also give appreciable absorption but without any marked peaks.

The separation of heptoses from other monosaccharides on paper chromatograms

TABLE I

MONOSACCHARIDE CONSTITUENTS OF "WALLS" OF ENCAPSULATED, SLIME-PRODUCING AND NON-ENCAPSULATED STRAINS OF *Klebsiella spp.*

Organism	*Strain*	*Preparation*	*Monosaccharides detected*
Kl. aerogenes	A3 capsulated	Cell walls	Galactose, glucose, fucose, uronic acid
		Wall extracted with hot water	Galactose, glucose
		Extracted capsular polysaccharide	Galactose, glucose, fucose, uronic acid
Kl. aerogenes	A3(Sl)	Cell walls	Galactose
Kl. aerogenes	A3(O)	Cell walls	Galactose
Kl. aerogenes	NCTC 8172	Cell walls	Galactose, glucose, mannose, rhamnose, uronic acid?, trace fucose?
Kl. cloacae	NCTC 5920	Cell walls	Glucose, rhamnose

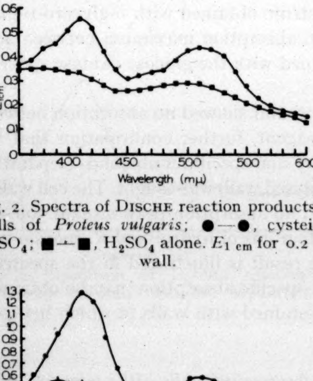

Fig. 2. Spectra of DISCHE reaction products of walls of *Proteus vulgaris*; ●—●, cysteine-H_2SO_4; ■—■, H_2SO_4 alone. $E_{1\,cm}$ for 0.2 mg wall.

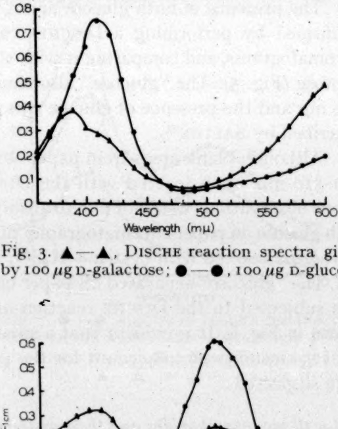

Fig. 3. ▲—▲, DISCHE reaction spectra given by 100 μg D-galactose; ●—●, 100 μg D-glucose.

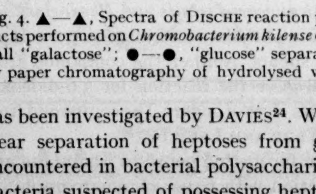

Fig. 4. ▲—▲, Spectra of DISCHE reaction products performed on *Chromobacterium kilense* cell-wall "galactose"; ●—●, "glucose" separated by paper chromatography of hydrolysed wall.

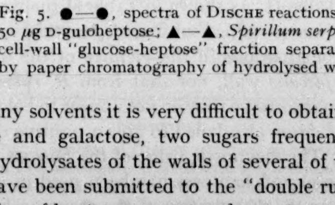

Fig. 5. ●—●, spectra of DISCHE reactions on 50 μg D-guloheptose; ▲—▲, *Spirillum serpens* cell-wall "glucose-heptose" fraction separated by paper chromatography of hydrolysed wall.

has been investigated by DAVIES[24]. With many solvents it is very difficult to obtain a clear separation of heptoses from glucose and galactose, two sugars frequently encountered in bacterial polysaccharides. Hydrolysates of the walls of several of the bacteria suspected of possessing heptoses have been submitted to the "double run" method used by DAVIES[24] for the identification of heptoses on paper chromatograms.

A satisfactory resolution of the heptoses of *Spirillum serpens* and *Proteus vulgaris* was not obtained and attempts to identify them by this means were abandoned until larger quantities of wall material would be available.

Confirmation of the presence of heptoses in the walls of several bacteria has been obtained by elution of the monosaccharides from paper chromatograms and subjecting them to the DISCHE reaction. As shown in Fig. 3, the conditions employed for the DISCHE test for heptoses also gives rise to coloured compounds for glucose and galactose. After hydrolysis and separation of the sugars in the wall of *Chromobacterium kilense*, the compounds corresponding to galactose and glucose on paper chromatograms were eluted and then subjected to the DISCHE reaction in the usual way. The absorption spectra for "glucose" and "galactose" presented in Fig. 4, both show the presence of material giving a peak between 500–510 mμ. No such absorption was detectable in a paper blank nor in the "mannose" separated on the same paper chromatograms. That the heptoses overlap the glucose and galactose was suspected from the pinkish colour of the chromatograms sprayed with aniline phthalate (aldohexoses give a brown colour[19], authentic heptoses gave a pink colour). Similar results were obtained with walls of *Chromobacterium prodigiosum* and *Photobacterium albensis*.

The presence of both glucose and a heptose in the wall of *Spirillum serpens* was confirmed by performing a DISCHE reaction on the "glucose" eluted from paper chromatograms, and comparing it with the spectrum obtained with D-glycero-D-gulo-heptose (Fig. 5). The "glucose" also showed an absorption maximum between 400–410 mμ and the presence of glucose was confirmed with the glucose oxidase spray as described by SALTON[21].

Although blank areas from paper chromatograms showed no absorption between 500–510 mμ when reacted with the DISCHE reagent, further confirmation that the above observations were not due to absorption by non-specific compounds (separating with glucose on paper chromatography of hydrolysed wall) was sought. The cell wall of *R. rubrum* reacted with cysteine–H_2SO_4 showed no absorption peak around 500–510 mμ. The "glucose" separated on paper chromatograms of hydrolysed *R. rubrum* wall was subjected to the DISCHE reaction and the result is illustrated in the spectrum shown in Fig. 6. It is evident that a small, non-specific absorption may be obtained, but it is insufficient to account for the peaks obtained with walls in which heptoses were suspected.

Cell-wall monosaccharides and their distribution after treating walls with various reagents

Cell walls of various Gram-negative bacteria have been examined by paper chromatography, combined with the DISCHE reaction of certain sugars separated on paper chromatograms as described in the preceding section. The results are presented in Table II. As shown in Table II the walls of *Alcaligenes faecalis* and *Rhodospirillum rubrum* contained substances reacting with aniline phthalate and migrating on paper chromatograms in the vicinity of the dideoxy sugars. Neither the compound from *A. faecalis* wall, nor that from *R. rubrum* wall gave the reaction for 3,6-dideoxy-aldohexoses, so they do not appear to belong to this class of sugars. The compound from *R. rubrum* also failed to give a positive direct WEBB AND LEVY test[25].

The diagnostic value of the DISCHE reaction for methyl pentoses (rhamnose and fucose) and heptoses has been used in combination with paper chromatography to follow the distribution of the monosaccharide constituents after subjecting the walls

TABLE II

MONOSACCHARIDE CONSTITUENTS OF WALLS OF GRAM-NEGATIVE BACTERIA

Walls from	*Monosaccharides detected*
Alcaligenes faecalis	Glucose, arabinose, fucose, rhamnose, unknown*
Bacterium cadaveris	Galactose, glucose, rhamnose
Chlorobium thiosulphatophilum	Galactose, glucose, mannose, rhamnose
Chromobacterium kilense	Galactose, glucose, heptose, mannose
Chromobacterium prodigiosum	Glucose, heptose, mannose, traces pentose (?) and rhamnose
Escherichia alkalescens	Galactose, glucose, rhamnose
Escherichia dispar	Galactose, glucose, rhamnose
Organism LC 1	Galactose, glucose, rhamnose
Photobacterium albensis	Glucose, heptose
Photobacterium fischeri	Galactose, glucose
Proteus vulgaris	Glucose, heptose
Pseudomonas sp.	Glucose, fucose, rhamnose
Rhodospirillum rubrum	Glucose, fucose, rhamnose, unknown*
Salmonella gallinarum	Galactose, glucose, mannose, rhamnose, tyvelose
Spirillum serpens	Glucose, heptose, rhamnose

* Both compounds migrating near dideoxy sugars, but give negative reactions for 2 and 3,6 deoxy sugars.

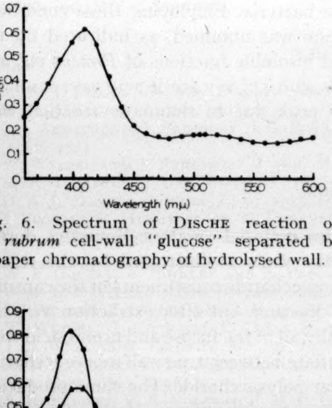

Fig. 6. Spectrum of DISCHE reaction on *R. rubrum* cell-wall "glucose" separated by paper chromatography of hydrolysed wall.

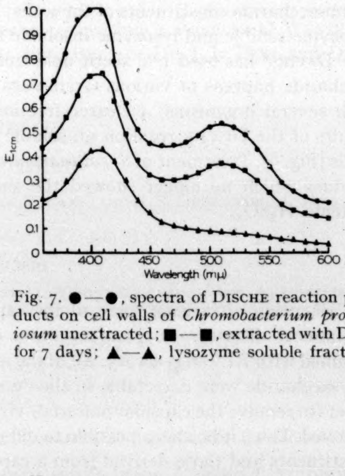

Fig. 7. ●—●, spectra of DISCHE reaction products on cell walls of *Chromobacterium prodigiosum* unextracted; ■—■, extracted with DEG for 7 days; ▲—▲, lysozyme soluble fraction.

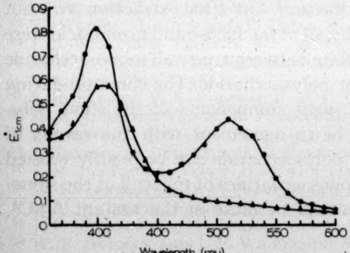

Fig. 8. ●—●, spectra of DISCHE reactions on *Spirillum serpens* untreated wall (1.5 μg); ▲—▲, lysozyme-soluble fraction (1.4 μg).

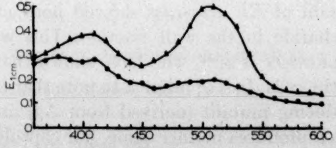

Fig. 9. Spectra of DISCHE reactions on cell walls of *Proteus vulgaris* treated with 1 % acetic acid, 4 h, 100°. ▲—▲, acetic acid "soluble" fraction; ●—●, insoluble residue.

to various extraction procedures. DEG, TCA and 1 % acetic acid have been used for the extraction of bacterial polysaccharides and lipopolysaccharide complexes[6, 26]. Cell walls of *S. gallinarum, Kl. aerogenes* A3 (O), *Chromobacterium prodigiosum* and *R. rubrum* were extracted with DEG for 1 week at room temperature. Soluble and insoluble fractions were separated and in all instances DISCHE reactions and paper chromatography showed the presence of all the monosaccharide constituents in both fractions. Extraction of walls of *S. gallinarum* with 10 % TCA (3 days at 20°) and with 90 % w/w phenol for 24 h at 37° also gave soluble products and insoluble residues containing all of the monosaccharides present in the parent wall material.

Although extraction with DEG failed to remove the polysaccharides quantitatively from the wall, leaving the characteristic sugars in the insoluble residues as well, treatment of walls with lysozyme (using conditions outlined previously[27]) did give a clearer fractionation of the walls of several organisms. The DISCHE reactions on the unextracted, DEG-extracted walls of *Chromobacterium prodigiosum* are compared with the lysozyme soluble fraction in Fig. 7. With *Spirillum serpens* the spectrum of the DISCHE reactions products again showed a marked difference between the original walls and the lysozyme-soluble fraction as shown in Fig. 8. However, treatment of the walls of *S. gallinarum* with lysozyme did not give a complete fractionation of the monosaccharide constituents of the walls; all sugars were again detectable in both the lysozyme-soluble and lysozyme-insoluble fractions.

DAVIES[6] has used 1 % acetic acid treatment for 4 h at 100° to release the polysaccharide haptens of various Gram-negative bacteria. Employing these conditions with several organisms, a clearer fractionation was obtained, as indicated by the results of the DISCHE reaction on soluble and insoluble fractions of *Proteus vulgaris* walls (Fig. 9). Treatment of *S. gallinarum* walls with 1 % v/v acetic acid gave insoluble residues which no longer showed the sharp peak due to rhamnose reaction with cysteine–H_2SO_4.

DISCUSSION

The retention of at least some of the capsular material on the cell surface during mechanical disintegration and isolation of the walls is illustrated by the results obtained with *Kl. aerogenes* A3. All of the monosaccharide constituents of the capsular polysaccharide were detectable in the "wall" fraction, but after extraction with hot water (to remove the capsular material) virtually all of the fucose and uronic acid were removed. Thus, it has been possible to differentiate between true wall monosaccharide constituents and those derived from a capsular polysaccharide. The slime-producing mutant of *Kl. aerogenes* showed none of the sugar components of the slime polysaccharide in the wall fraction. This would be in agreement with the results of WILKINSON *et al.*[22], who have shown that the polysaccharide can be readily washed off the cells. It is of interest to note that the monosaccharides of the wall of the slime-producing mutant (derived from A3) are identical to those of the mutant A3(O), which produces neither slime, nor capsule.

Attempts to obtain a clear-cut fractionation of the monosaccharide constituents of the walls by extraction with DEG have not been very successful. BROWN[28] has also shown that extraction of walls with TCA removes material of fairly similar composition to what is left in the insoluble fractions of the walls. Lysozyme treatment on the other hand has conclusively established the presence of heptoses in the lipo-polysaccharide-protein fraction of the wall and not in the mucopeptide part which is released into solution by lysozyme action[5]. 1 % acetic acid treatment for 4 h at 100° has also given a fairly selective release of the sugar components.

The results of these investigations are in accord with the view that the polysaccharides characteristic of the Smooth and Rough antigenic constituents of the bacterial cell are an integral part of the lipid-polysaccharide-protein fraction of the bacterial wall and some of the polysaccharide may even be linked to the mucopeptide material released by lysozyme (as with *S. gallinarum*). Thus, the description of the "surface" antigenic polysaccharide complexes as "micro-capsular"[13,14] constituents of the bacterial cell is misleading and should be discontinued. Whether the polysaccharide complexes are localized in a particular layer of the multilayered wall of a Gram-negative organism is not known. Preparation of ultra-thin sections of walls subjected to the more selective of the extraction procedures used in this study should greatly clarify an understanding of the structure of the walls of the Gram-negative bacteria.

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Short Communications

Separation of calf-lens proteins by means of vertical-column zone electrophoresis*

The classical concept of the protein composition of the crystalline lens is that of four different fractions, one insoluble, albuminoid, and three soluble, α-, β-, and γ-crystallin. However, recent investigations employing immunological and agar microelectrophoretical methods have shown that the protein composition of the lens is more complicated than was previously believed[1–3]. Consequently new methods must be applied to the purification of the different proteins, for which purpose chromatography on cellulose ion exchangers seems to be very promising. A preliminary report on the separation of lens proteins on DEAE-cellulose has already been published[4]. However, the complexity of the chromatographic pattern makes a suitable prefractionation method highly desirable. For this purpose the preparative zone-electrophoretic procedure of PORATH has been applied to the lens proteins. This method was found to have higher capacity and to give better resolution than the starch-block electrophoresis technique used by BLOEMENDAHL AND TEN CATE[5].

Materials and methods

Calf eyes were removed 30–60 min after the death of the animal and immediately put on ice. The lenses were removed, freed from the capsule and homogenized in a Potter-Elvehjem homogenizer in an 0.05 *M* Tris buffer, pH 8.0 (5 ml per 3 lenses). The homogenate was centrifuged for 45–60 min at 15,000 × *g*, and the supernatant was dialyzed for 24 h against three changes of 750 ml 0.05 *M* Tris buffer (pH 8.0). All preparative procedures were conducted at 4°.

Vertical-column zone electrophoresis with cellulose as supporting medium was used for the separation of the proteins[6,7]. The column, 3.2 × 100 cm, was packed with cellulose powder (Munktell AB, Sweden), suspended in Tris buffer. The packing was carried out with a positive pressure of 25–30 lb./in.^{2}. The void volume of the column was 610 ml as tested with a color band. 12 ml of protein solution with a concentration of approx. 8 % was introduced 15 cm into the column at a rate of 3–4 cm/h to compensate for the electroendosmotic flow. The electrophoresis was performed in 0.05 *M* Tris buffer (pH 8.0) with a potential gradient of 5 V/cm for 46 h at 0.5°. The current was approx. 45 mA. To obtain optimum separation it was found necessary to treat the column with 0.2 *M* NaOH between runs.

The column was eluted at 4° with a flow rate of 20 ml/h. The eluate was collected in 5-ml fractions, the absorbancies of which were measured at 280 mμ in a Beckman DU spectrophotometer.

The pooled protein fractions were concentrated by ultrafiltration through

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Abbreviations: DEAE-, diethylaminoethyl-; Tris, tris(hydroxymethyl)aminomethane.

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collodion shells (Schleicher and Schüll, Keene, New Hampshire), and then dialyzed for 24 h against three changes of the buffer used in the following experiments.

Free electrophoresis experiments were performed in veronal–acetate buffer, I = 0.15, at 1° in an Aminco Portable Electrophoresis Apparatus using a 3-ml Tiselius cell. Ultracentrifugation was done at room temperature in a Spinco Model E analytical ultracentrifuge at 52,640 rev./min in a standard 4°-sector cell. The buffer used was 0.1 *M* phosphate (pH 6.9).

Protein concentrations were measured by the method of LOWRY *et al.*[8] with human serum as standard.

All buffers contained 10 % (v/v) of a saturated solution of 5.7-dichloro-8-quinolinol as a chelating agent.

Results and discussion

The zone electrophoretic separation is shown in Fig. 1. Three distinct fractions, denoted as Fractions I, II and III, were obtained. The pattern is reproducible, if the column is treated with NaOH between runs. An untreated column causes the disappearance of the third peak, probably because of an increased electroendosmotic flow. The recovery was approx. 85 % in all experiments. Evidently some adsorption of the protein to the cellulose occurred, which explains the large elution volume of Fraction III.

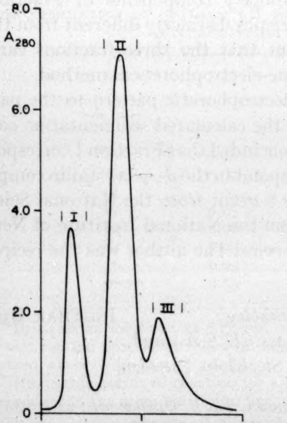

Fig. 1. Zone electrophoresis of calf-lens proteins in 0.05 *M* Tris buffer (pH 8.0); 46 h at 5 V/cm. Proteins with the highest mobility towards the anode are eluted first. The vertical lines indicate those parts of the fractions used for further analyses.

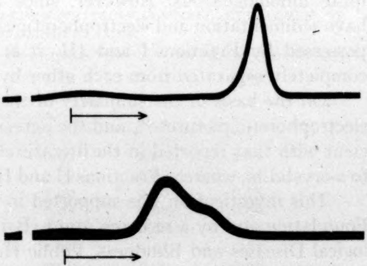

Fig. 2. Free electrophoresis patterns in veronal–acetate buffer, I = 0.15, pH 8.2. Current 8 mA, ascending boundaries; migration towards the anode. A: Fraction I, 21 mg/ml, 270 min, bar angle 20°. B: Fraction II, 15 mg/ml, 420 min, bar angle 45°. The vertical lines indicate the initial position of the boundaries and the arrows the direction of migration.

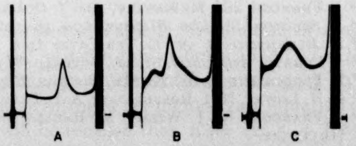

Fig. 3. Ultracentrifugation patterns at 52,640 rev./min in 0.1 *M* phosphate buffer (pH 6.9). A: Fraction I, 12 mg/ml, 24 min, phase-plate angle 60°. B: Fraction II, 24 mg/ml, 39 min, phase-plate angle 50°. C: Fraction III, 13 mg/ml, 167 min, phase-plate angle 35°. The times were measured after reaching full speed.

The fractions were pooled as indicated in Fig. 1, concentrated, dialyzed and subjected to electrophoretical and ultracentrifugational analysis.

The free-electrophoresis results are shown in Fig. 2. As can be seen, both Fractions I and II are negatively charged at pH 8.2. Fraction I is electrophoretically homogeneous at this pH, whereas Fraction II shows two peaks. Fraction III exhibited very low mobility at both pH 8.2 and 6.6. However, the protein partly precipitated in the electrophoresis cell during the run at the concentration and temperature used. The precipitate redissolved to a large extent at 6°. Because of this fact no definite conclusion can be drawn at present as to the electrophoretical homogeneity of Fraction III.

The ultracentrifuge patterns are shown in Fig. 3. Fraction I and III sediment as single components, but Fraction II shows considerable heterogeneity. In addition to the main peak it contains two smaller components, one with higher and the other with lower sedimentation velocity.

The sedimentation coefficients of Fraction I, which was both electrophoretically and ultracentrifugationally homogeneous, were measured at different concentrations in 0.1 *M* phosphate buffer (pH 6.9). By extrapolation of these values to infinite dilution a value for $S_{20,w}$ of 18,3 ± 0.2 was found.

Fraction I is the only fraction that is known to be homogeneous as judged from both electrophoresis and ultracentrifugation. Fraction III is homogeneous in the ultracentrifuge, but its electrophoretical homogeneity is uncertain. Fraction II is quite inhomogeneous. However, since the subsidiary components of Fraction II have sedimentation and electrophoretic characteristics distinctly different from those possessed by Fractions I and III, it is apparent that the three fractions can be completely separated from each other by the zone-electrophoretical method.

On the basis of the similarity of the zone-electrophoretic pattern to the paper-electrophoretic picture[9, 10], and the agreement of the calculated sedimentation coefficient with that reported in the literature[11], it is concluded that Fraction I corresponds to α-crystallin, whereas Fractions II and III correspond to the β-, γ-crystallin complex.

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The kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-N-(acetylglycyl)-L-tyrosinhydrazide

The lack of unambiguous kinetic constants for the α-chymotrypsin-catalyzed hydrolysis of substrates of the type RCONHCHR′CONHCHR″COY, where –COY is the carboxyl derivative undergoing solvolysis, led us to investigate the kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-N-(acetylglycyl)-L-tyrosin-hydrazide in aqueous solutions at 25° and pH 7.80 and 0.02 M in the Tris component of a Tris–HCl buffer. This reaction system was selected for study because (a), information was needed for the case of R′ = H before proceeding to substrates containing two or more centers of asymmetry; (b), it could be followed by a convenient and unambiguous analytical procedure[1,2], and (c), the kinetic constants could be compared with those evaluated earlier for a comparable system involving α-N-acetyl-L-tyrosinhydrazide[1,2], a closely related substrate of the type RCONHCHR′COY.

The selection of pH 7.80, as one of the reaction parameters, was not arbitrary but was based upon consideration of the data given in Table I. It was concluded that the optimum pH for the reaction system was 7.80 ± 0.05, a value identical, within the limits of error, with that determined for the comparable system containing α-N-acetyl-L-tyrosinhydrazide, *i.e.*, 7.95 ± 0.20 (see refs. 1 and 2).

TABLE I

DETERMINATION OF OPTIMUM pH FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-(ACETYLGLYCYL)-L-TYROSINHYDRAZIDE*

pH	7.33	7.73	7.85	8.10	8.27
time (min)	*Relative extent of reaction***				
4	0.90	0.96	0.99	1.00	0.85
6	0.92	0.92	1.00	0.90	0.85
8	—	0.98	1.00	0.90	0.78
10	0.93	1.00	0.95	0.88	0.88
12	0.96	0.97	1.00	0.92	0.88
14	0.93	1.00	1.00	0.91	0.85
16	0.93	1.00	0.99	0.90	0.88
mean	0.93	0.98	0.99	0.92	0.85

* In aqueous solutions at 25° and 0.02 M in the Tris component of a Tris–HCl buffer with $[E] = 0.140$ mg protein nitrogen/ml and $[S]_0 = 4.15 \cdot 10^{-4}$ M. Essentially the same results were obtained when $[S]_0 = 10.37 \cdot 10^{-4}$ M.

** Maximum extent of reaction for any given reaction time normalized to a value of 1.00. All other values in the same horizontal line refer to fractional extent of reaction relative to maximum.

The data from 26 experiments performed in order to evaluate the constants K_S and k_3 for the system of interest are summarized in Table II. Evaluation of these data, by the method of BOOMAN AND NIEMANN[3], using in this instance a DATATRON 205 digital computer for the actual computations, gave values of $K_S = (22 \pm 9) \cdot 10^{-3}$ M and $k_3 = (1.1 \pm 0.4) \cdot 10^{-3}$ M/min/mg protein nitrogen per ml for a rate equation of the form $dP/dt = k_3[E][S]/(K_S + [S])$. It will be recalled that $K_S = (29.5 \pm 6.0) \cdot 10^{-3}$ M and $k_3 = (1.1 \pm 0.2) \cdot 10^{-3}$ M/min/mg protein nitrogen per ml for the comparable system involving α-N-acetyl-L-tyrosinhydrazide[1,2].

Abbreviation: Tris, tris(hydroxymethyl)aminomethane.

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It is evident that the constants K_S and k_3 for the systems α-chymotrypsin–α-N-(acetylglycyl)-L-tyrosinhydrazide and α-chymotrypsin–α-N-acetyl-L-tyrosinhydrazide, in aqueous solutions at 25° and pH 7.8 ± 0.2 and 0.02 M in the Tris component of a Tris–HCl buffer, are identical within the limits of experimental error. The implication that replacement of CH_3CONH– by $CH_3CONHCH_2CONH$– is without significant effect upon the course of the reaction is surprising in view of the marked variation in values of K_S and k_3 observed in the series, α-N-formyl-, α-N-acetyl-, α-N-dichloroacetyl- and α-N-trimethyl-acetyl-L-tyrosinhydrazide[1]. However, with the present demonstration of the kinetic equivalence, or near equivalence, of α-N-(acetylglycyl)- and α-N-acetyl-L-tyrosinhydrazide we may infer that the additional carboxamide function, present in the former substrate, does not influence the course of the reaction either by an added steric interaction or by a supplementary binding interaction unless one is faced with the rather improbable situation that both such interactions occur but that they kinetically nullify each other. Thus, it appears that α-N-(acetylglycyl)-L-tyrosinhydrazide is a reasonable point of departure for

TABLE II

KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-(ACETYLGLYCYL)-L-TYROSINHYDRAZIDE*

$[S]_0$ × 10^4 M	Δt min**	v_0*** $A/min \times 10^3$	P_n****
0.765	6	2.29 ± 0.05	2
0.84	2.5	2.44 ± 0.06	2
1.39	4	3.36 ± 0.07	1
1.95	3	5.59 ± 0.10	2
2.79	3	8.29 ± 0.26	2
2.79	2.5	7.19 ± 0.14	1
3.72	4	9.43 ± 0.29	1
5.57	2	15.64 ± 0.32	1
5.99	3	16.43 ± 0.36	2
8.36	2	22.81 ± 0.30	2
8.69	4	25.68 ± 0.67	2
11.15	1	31.50 ± 0.68	1
11.98	3	36.02 ± 0.69	3
12.41	4	34.77 ± 0.41	2
12.53	1	34.97 ± 0.70	1
13.93	1	36.12 ± 1.05	1
15.52	1	40.60 ± 0.56	1
16.72	1	45.08 ± 3.15	1
17.96	3	47.69 ± 0.97	2
18.11	1	56.33 ± 0.68	3
19.50	[illegible]	49.72 ± 1.73	1
20.34	3	53.00 ± 0.95	2
20.90	1	51.57 ± 1.80	1
22.29	1	55.70 ± 1.63	1
23.95	3	56.99 ± 0.19	3
24.82	4	60.48 ± 0.61	3

* In aqueous solutions at 25° and pH 7.80 and 0.02 M in the Tris component of a Tris–HCl buffer with $[E] = 0.140$ mg protein nitrogen/ml.

** Time interval between successive determinations of amount of liberated hydrazine. For each experiment eight determinations were made including the one for $t = 0$.

*** The initial velocity, v_0, is given in units of absorbancy (A)/min.

* Degree of polynomial required to describe dP/dt^3.

the exploration of the kinetic properties of substrates of the type RCONHCHR′-CONHCHR″COY, where R = CH_3—, R′ ≠ H, R″ = p-$CH_2C_6H_4OH$ and Y = –$NHNH_2$, *i.e.*, those substrates containing two centers of asymmetry.

Experimental

L-Tyrosine (10 g) was acylated with chloroacetyl chloride[4] to give crude α-N-chloroacetyl-L-tyrosine which was allowed to react for 72 h with 150 ml 28 % aq. ammonia. The reaction mixture was evaporated to dryness and the residue recrystallized from ethanol to give glycyl-L-tyrosine hydrochloride. The dipeptide hydrochloride was suspended in 75 ml methanol, the mixture saturated with dry HCl, allowed to stand for 24 h at room temperature, the solvent and excess HCl removed *in vacuo* and the residue dried *in vacuo* over solid NaOH to give 7.4 g glycyl-L-tyrosine methyl ester hydrochloride. The latter product was taken up in 50 ml water containing 4.3 g anhyd. $NaHCO_3$ and covered with 100 ml ethyl acetate. Acetic anhydride, 2.6 g, was added in several portions and with vigorous shaking. The ethyl acetate phase was collected, dried over anhyd. Na_2SO_4 and evaporated to dryness. The syrupy residue was taken up in 25 ml anhyd. ethanol and the solution heated to its refluxing temperature prior to the dropwise addition of 2 ml of hydrazine hydrate. The reaction mixture was allowed to stand at room temperature overnight, the colorless precipitate collected, washed successively with absolute ethanol and ethyl ether, dried *in vacuo* and recrystallized twice from water to give 1.3 g α-N-(acetylglycyl)-L-tyrosinhydrazide, m.p. 244.0–245.0°, corr.

Anal. Calcd. for $C_{13}H_{18}O_4N_4$(294): C, 53.0; H, 6.2; N, 18.9. Found: C, 53.1; H, 6.2; N, 19.0.

The procedure employed for the kinetic studies was identical with that described previously[2]. The actual experimental parameters are given in Table II. The enzyme preparation was crystalline salt-free α-chymotrypsin, Armour Lot No. 00592.

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The calculation of kinetic constants of enzyme-catalyzed reactions using digital computers

A program has been written for the DATATRON 220 digital computer that will calculate, by means of a previously described orthogonal polynomial procedure[1,2], the so-called initial velocities of enzyme-catalyzed reactions, use these velocities, in conjunction with other known experimental parameters, to calculate kinetic constants, test the data, using the "t" test[3] to detect possible invalid points, and recalculate the constants after excluding such points. While the program, currently in use in these laboratories, is specifically designed for evaluation of the constants K_S and k_3 for systems exhibiting simple Michaelis-Menten kinetics, *e.g.*, those described by the equation $[S]_0[E]_0/v_0 = K_S/k_3 + [S]_0/k_3$, it can be readily adapted to other kinetic forms.

The program requires the introduction of nine chart coordinates of a trace of extent of reaction *vs.* time, taken at equal time intervals, the initial enzyme and substrate concentrations, the conversion factors for the chart scales, and the constants for the enzyme and substrate "blank" reactions. On introduction of a suitable command, the coefficients of the "least squares" curves of orders one through five are determined. The lowest, *i.e.*, the first-order coefficient, is then tested for significance according to the 90 % "t" test. If the coefficient is found to be significantly non-zero, the next higher coefficient, C_1, is tested by the same criterion and the process continued until the order of curvature, M, is found such that C_n, $n \leqslant M$ are significant and C_{M+1} is insignificant. The initial velocity is determined as before[1] from the initial slope of the curve of degree M, is corrected for the "blank" reactions and the quantities $y = [S]_0[E]_0/v_0$ and $x = [S]_0$ calculated and stored in appropriate memory locations. A tally is kept of the number of initial velocities evaluated and the x and y values for consecutive runs are introduced into consecutive memory locations.

When from 4 to 40 such calculations have been completed, and upon introduction of a suitable command, a linear least-squares fit of y *vs.* x is performed and values of K_S and k_3 computed from the apparent intercept and slope. The intercept, a, the slope, b, k_3 and K_S, and their respective uncertainties, x_i, y_i, $y'_i = a + bx_i$ and $d_i = y'_i - y_i$, for $i = 1$ to n are printed out.

The "worst point", *i.e.*, the one with the largest value of Z_i, where

$$Z_i = | d_i / \{ [(n+1)/n] \sum_{j=1}^{n} (x_j - \bar{x})^2 + (x_i - \bar{x})^2 \}^{\frac{1}{2}} |$$

and $\bar{x}$ is the average value of x, is chosen and the x and y values of this point removed from the active data and stored in a special memory location. A new least-squares fit is performed and the 98 %-confidence region about the new relationship is determined from the equation

$$A = t_{(0.98,\, n-2)} [\sum_{j=1}^{n} d_j^2/(n-2)]^{\frac{1}{2}} \{ [(n+1)/n] \sum_{j=1}^{n} (x_j - \bar{x})^2 + (x_i - \bar{x})^2 \}^{\frac{1}{2}} ,$$

where $t_{(0.98,\, n-2)}$ is the 98 % "t" coefficient for n--2 degrees of freedom. A is the interval along the y axis such that the probability that $(a + bx_i - A) < y_i < (a + bx_i + A)$ is 98 %, provided (x_i, y_i) is a member of the family of normally distributed points used to calculate a and b. The y coordinate of the "worst point" is then examined and if it lies within the required interval, the computer is placed in a ready state for the introduction of new data. If the point lies outside the interval, the results based upon the new relationship are printed out and a new "worst point" is chosen and tested as above. This process is continued until the "worst point" passes the significance test or the data is reduced to three points.

Access can be made to each section of the program without involving any other. Velocities can be calculated without determination of kinetic constants and the constants can be evaluated from predetermined velocities. The program can also be keyboard modified to compute velocities according to any desired order of curvature, from one to five, rather than relying upon a significance test. On high-speed-tape output, each velocity calculation requires approx. 15 sec. Most of the time is used by the extensive output and this can be simplified if time of operation is a serious consideration. The *y vs. x* least-squares fit requires 15 sec for each iteration.

The preceding program was developed from an earlier one written for the DATATRON 205 computer, a model of lesser capability than the 220. Aside from differences arising from the different characteristics of the two computers the two programs were similar up to the point of obtaining initial velocities. However, in contrast to the 220 program, which employs a point by point significance test in the evaluation of K_S and k_3, the 205 program determined the confidence region about the initial least-squares fit of *y vs. x* and discarded all points lying outside this region before proceeding to a second and final least-squares fit. The 205 program also was unable to accept initial-velocity data obtained at more than one enzyme concentration and lacked self-clearing procedures which in the 220 program allows a virtually unlimited number of experiments to be evaluated without recycling the program tape.

One feature of the 205 program, which was not incorporated in the 220 program, involving a stepping procedure which first calculated a value of the initial velocity using n equally spaced time coördinates and then repeated the calculation for $n-1$ coordinates dropping the initial or $t = 0$ point. While this procedure is useful for detecting an aberrant initial point, generally arising from operational difficulties, it was not included in the 220 program because the same information can be obtained in the majority of cases by direct examination of the trace of extent of reaction *vs.* time. In those cases where it cannot, it is a simple matter, with the 220 program, to introduce a second set of data excluding the initial point.

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Change of helical configuration in the activation of chymotrypsinogen

Since it has been demonstrated by DESNUELLE *et al.*[1] and NEURATH *et al.*[2] that trypsinogen and chymotrypsinogen are activated only through the cleavage of one peptide linkage, it is interesting to investigate the intramolecular changes of configuration accompanying this activation process. In fact, NEURATH *et al.*[3] found that the levorotation decreased markedly during this process. They thus came to a conclusion that the degree of helical formation was increased by the activation, giving rise to a configuration suitable for forming the active center.

Ingenious as it appears, this work of NEURATH *et al.* involves some serious uncertainties in drawing decisive conclusions. First of all, they determined only the specific rotation and not the rotatory disperion. As has been pointed out by one of the authors, denaturation of proteins into the corresponding β-forms is accompanied by a rise in the specific rotation[4]. Therefore, the decrease of levorotation does not necessarily reflect the increase of the helical fraction. Moreover, the presence of liberated peptides, especially in the case of trypsinogen, might also have contributed to the change of the specific rotation accompanying the reaction.

To exclude these ambiguous points, we compared the rotatory dispersion of chymotrypsinogen with that of π-chymotrypsin which was isolated from the former in a pure state. Chymotrypsinogen was prepared from beef pancreas according to the method of KUNITZ AND NORTHROP[5]. After being recrystallized several times in the presence of 10^{-3} *M* di-isopropylfluorophosphate and lyophylized, the sample showed less than 0.05 % of chymotrypsin activity and 0.05 % trypsin activity. π-chymostrypsin was prepared by activation (at 0° and pH 7.7) of the chymotrypsinogen with 1/50th its weight of trypsin in the presence of β-phenylpropionic acid. After complete activation, the mixture was dialysed against an HCl solution of pH 3 and lyophilized. The specific activity of this sample was 4.5 when measured with L-acetyltyrosine ethyl ester as substrate. Good homogeneity of chymotrypsinogen and π-chymotrypsin prepared was ascertained also by the patterns of their elution chromatograms.

Before carrying out the examination of rotation the existence of the β-configuration in the samples was checked by measuring the infrared absorption spectrum at 1910 cm^{-1} which corresponds to the $>$C=O stretching frequency of the β-configuration[6]. With both samples the results were negative so that the existence of β-fraction could be left out of consideration. In the absence of β-configuration, the data of rotatory dispersion of proteins can be expressed by the following empirical formula[4]

$$\frac{\lambda^2-\lambda_0^2}{\lambda_0^2}[m'] = a_0^R + f^H a_0^H + \frac{\lambda_0^2}{\lambda^2-\lambda_0^2} f^H b_0^H \qquad (1)$$

where λ and λ_0 are wavelength and characteristic wavelength, respectively, a_0^R the intrinsic residue rotation, $[m']$ the effective residue rotation, f^H the fraction of the helical part, and a_0^H and b_0^H are contributions of helical configuration, which have been determined to be +680 and —600, respectively, from the data of synthetic polypeptides. Effective residue rotation $[m']$ can be obtained from the specific rotation $[\alpha]$ by the equation:

$$[m'] = \frac{M}{100}\,\frac{3}{n^2+2}\,[\alpha] \qquad (2)$$

where M denotes the mean residue weight and n the refractive index of the solvent.

In Fig. 1 are shown the rotatory dispersions measured for both samples with the assumption that λ_0 is 212 mμ. Since a_0^R of chymotrypsinogen and π-chymotrypsin has been found from the dispersion in 8 M urea (in which $f^H = 0$) to be −580, the helical content f^H could be determined from the slopes and intercepts of the curves in Fig. 1. The results obtained are shown in Table I, from which it can be seen that the activation of the zymogen is accompanied by a 2-fold increase of the helical content. Since the molecular weights of chymotrypsinogen and π-chymotrypsin are about 24,000, it is concluded that a helical fraction of 5 to 6 turns is formed by the activation. It is noteworthy that such a small fraction is essential for the manifestation of the activity of this enzyme.

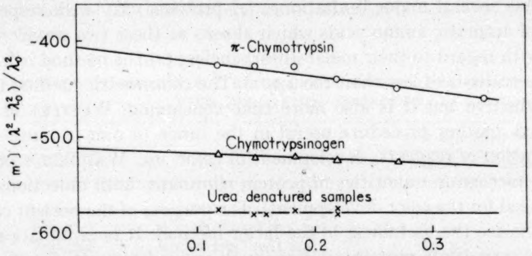

Fig. 1. Fit of equation (1) to the results of measurement of rotation. Medium for chymotrypsinogen and π-chymotrypsin: 0.05 M phosphate buffer (pH 7.7) containing 0.1 M β-phenylpropionate. For urea-denatured samples of both preparations: 8 M urea, pH 7.7.

TABLE I

HELICAL CONTENT OF NATIVE AND UREA-DENATURED CHYMOTRYPSINOGEN AND π-CHYMOTRYPSIN

	$-[\alpha]D$	$-f^H b_0^H/600$	$f^H a_0^H/680$
Chymotrypsinogen	87.4	0.12	0.11
π-chymotrypsin	74.3	0.23	0.26
Urea-denatured chymotrypsinogen	105.0	0.00	0.00
Urea-denatured π-chymotrypsin	104.3	0.00	0.00

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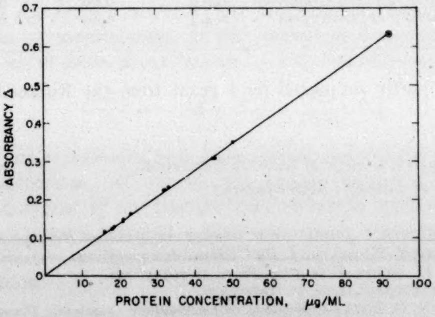
Note on spectrophotometric determination of proteins in dilute solutions

One of the most common methods of measuring protein concentrations involves determination of the absorption of the protein solution at 260–280 mμ[1]. While useful, this procedure has several major limitations: (1) proteins vary with respect to the concentrations of aromatic amino acids which absorb at these two wavelengths and therefore vary with regard to their molar absorbancies; (2) the method is insensitive to protein concentrations of less than 100 μg/ml. The colorimetric method of LOWRY *et al*[2] is more sensitive but it is also more time consuming. WESTLEY *et al*[3] have recently proposed another procedure useful in the range of 0.01–1.0 mg but also requiring preparation of reagents, development of color, etc. WADDELL'S procedure[4] for determining microgram quantities of protein eliminates both objections (lack of sensitivity and need for the color development). The purpose of the present communication is to emphasize the usefulness of the latter method. It is rapid, accurate and can be used with very dilute protein solutions such as are frequently encountered in chromatographic separations. SPIES *et al*[5] have also shown that absorption in this region of the u.v. is proportional to protein concentration. BENDIXIN[6] has reported that WADDELL'S procedure for blood and cerebrospinal fluid proteins compares satisfactorily with either Kjeldahl nitrogen values or LOWRY'S colorimetric method.

WADDELL'S method has been used routinely in this laboratory for some time. WARBURG AND CHRISTIAN'S method[1] for determining protein concentrations from the 280 mμ/260 mμ absorbancy ratio could not be used with solutions of brain proteins, perhaps because of their low aromatic amino acid contents. In addition, the latter method was not sensitive enough for following chromatographic fractionation of small quantities of protein. In order to demonstrate the usefulness of WADDELL'S procedure, protein solutions made from a Versatol** standard have been utilized for much of the data presented. A Beckman DU spectrophotometer was used to measure the absorbancy of these solutions at 215 and 225 mμ.

As seen in Fig. 1, Beer's Law was obeyed over a concentration range of 20–100 μg/ml. We have obtained similar results using either albumin or a guinea-pig-brain



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** Obtained from Warner-Chilcott, Laboratory Supply Division, New York, N.Y.

protein as standards. The ordinate in Fig. 1, *i.e.*, absorbancy Δ, is the difference between the absorbancies at 215 and 225 mμ.

Table I lists the various buffers which have been found to be satisfactory. Because of the high absorption at 215 mμ, acetate, succinate, citrate, phthalate and barbiturate buffers cannot be used in 0.1 *M* concentration. With proper blanks, 0.005 *M* solutions of all these buffers are satisfactory.

TABLE I

SATISFACTORY BUFFERS FOR DETERMINATION OF PROTEIN CONCENTRATION FROM 215–225 mμ ABSORPTION

Protein	*Buffer*	*Protein concentration (mg/ml)*	
		Weight	*u.v. data*
Versatol	0.1 *M* borate (pH 9.8)	10	10.1
Versatol	0.1 *M* phosphate (pH 7.2)	10	9.1
Versatol	0.1 *M* tris* (pH 8.8)	10	9.2
Versatol	0.005 *M* acetate (pH 5.1)	10	9.2
Versatol	0.005 *M* succinate (pH 6.3)	10	9.1
Albumin	0.01 *N* HCl	10	9.4
Brain protein	0.1 *M* phosphate (pH 7.3)	13.5	13.7

* Tris(hydroxymethyl)aminomethane.

STRICKLAND *et al.*[7] have said that this method was not satisfactory when applied to electrophoretically separated fractions of serum proteins. Their protein fractions were dissolved in 0.1 *N* NaOH which cannot be used as a solvent in WADDELL's procedure because of its high absorption in this region of the spectrum. This objection while valid for solutions which contain 0.1 *N* NaOH would not apply if 0.005 *N* alkali was used (Table II). NaCl and $(NH_4)_2SO_4$ solutions commonly encountered in protein-fractionation procedures do not interfere. This method has been utilized quite successfully in following the chromatographic separation of guinea-pig-brain proteins on CM-cellulose columns. For eluting, a NaCl gradient (0–1.0 *M*) was used in 0.005 *M* sodium acetate buffer (pH 5.1).

This spectrophotometric method has great advantages over other procedures in simplicity, rapidity and sensitivity. Since $(NH_4)_2SO_4$ does not interfere with the determination, it would be particularly useful in following salt fractionations.

The only observed limitation of the method is that many salts and acids commonly present in protein solutions absorb in this region of the spectrum. This disad-

TABLE II

DETERMINATION OF PROTEIN CONCENTRATION BY U.V.-ABSORPTION; COMPARISON OF WADDELL'S METHOD WITH THE TECHNIQUE OF WARBURG AND CHRISTIAN

Protein	*Buffer*	*Protein concentration (mg/ml)*		
		Weight	WARBURG AND CHRISTIAN[1]	WADDEL[4]
Albumin	0.005 *M* acetate	18	14.7	17.5
Albumin	0.005 *N* NaOH	18	10.1	17.8
Albumin	0.1 *M* borate	18	11.7	17.5

vantage can be overcome by substituting a solute which does not absorb (see Table I) or by using such dilute solutions that the buffer solution alone can be used as a blank. Because of the sensitivity of the method, extreme care must be taken in preparing the dilutions of the unknown solution.

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The heterogeneity of ovomucoid

This report concerns the behaviour of some ovomucoid preparations on gradient elution from columns of triethylaminoethylammonium-cellulose. In all the four preparations examined, which were obtained by using trichloroacetate to precipitate other egg-white proteins[1,2], it was possible to show the presence of substantial impurities of unrelated proteins, in agreement with the results of an investigation by zone electrophoresis[3]. Indications were also obtained of some degree of heterogeneity of the ovomucoid itself.

The results of one run are shown in Fig. 1. Of the three main peaks emerging in succession as the NaCl concentration is increased, only the second showed antitryptic

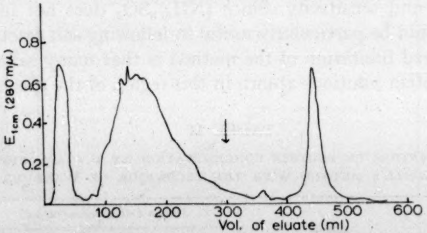

Fig. 1. Chromatography of ovomucoid (preparation B of Table I) on triethylaminoethylammonium-cellulose (Serva Entwicklungslabor, Heidelberg, Germany). The sample (20 ml, E_{1cm} 6.5 at 280 mμ, previously dialysed free of salts) was applied to a column 4.5 cm high and 2 cm diameter, equilibrated before use with 0.004 *M* sodium phosphate buffer (pH 6.8). Elution was effected first by gradient to 0.2 *M* NaCl, 0.004 *M* phosphate (pH 6.8); beginning at the point indicated by the arrow, a second gradient to 1.0 *M* NaCl, 0.004 *M* sodium phosphate (pH 6.8) was applied. The eluate was collected in fractions of 3.4 ml at a rate of 0.3 ml/min. Temperature, 2°.

activity[4] and appreciable amounts of neutral sugar[5]. The first and third peaks therefore contained contaminating materials different from ovomucoid, presumably protein in nature, since they showed absorption maxima near 280 mμ. "Deproteinising" by the SEVAG procedure[6] selectively removed the material of the first peak. The third peak varied most in quantity from one preparation to another (Table I).

TABLE I

CHROMATOGRAPHIC COMPONENTS OF OVOMUCOID PREPARATIONS*

*Preparation***	*A*	*B*	*B****	*C*	*D*
First peak	9	11	1	16	14
Second peak	71	49	62	52	57
Third peak	5	12	9	10	3

* As % of total material applied to column, measured by absorption at 280 mμ. Chromatographic conditions as in Fig. 1.

** Preparations A, B and C were made from fresh hen's egg white by addition of sodium trichloroacetate to a concentration of 5 % at pH 3.5, followed by precipitation of the ovomucoid from the supernatant by 2 vol. 95 % ethanol at 0° and pH 6.0[2]. Preparation D was bought from L. Light and Co, Colnbrook, England, and stated to have been obtained by the trichloroacetic acid–acetone method[1].

*** "Deproteinized" before chromatography by twice shaking overnight at room temperature with 0.25 vol. *n*-pentanol–chloroform (3:1, v/v)[6].

Some degree of heterogeneity of the ovomucoid itself was suggested not only by the broad and asymmetric shape of the second peak, but also by a partial resolution into components in the case of preparation C (Table I), the E_{1cm} at 280 mμ falling to 0.15 at an eluate volume of 143 ml between peaks of 0.28 at 122 ml and 0.48 at 170 ml. After each run, therefore, the fractions composing the early, middle and late parts of the ovomucoid peak were pooled separately to give three approximately equal portions. These pooled fractions were then compared with respect to the following properties:

(*a*) *Antitryptic activity*. The residual trypsin activity, measured[4] with *p*-toluenesulphonyl-L-arginine methyl ester as substrate, was determined as a function of the amount of inhibitor added, expressed in terms of its absorption at 280 mμ; the inhibitory potencies thus found differed by less than the experimental error of approx. 5 % for the different fractions.

(*b*) *Content of neutral sugars*, determined by the absorption at 500 mμ developed with an orcinol–H_2SO_4 reagent[5]. Using solutions adjusted to the same E_{1cm} at 280 mμ, the results for the different fractions differed by less than 5 %.

(*c*) *Nature of the neutral sugars present*. After hydrolysis in 1.0 *N* HCl at 95° for 16 h, the HCl and the bulk of the amino acid and peptide hydrolysis products were absorbed on a column of the anion-exchange resin Amberlite IRA 400 in the bicarbonate form. The sugars remaining in solution were subjected to paper chromatography, using butanol–pyridine–water (6:4:3, v/v/v) as moving phase and aniline hydrogen phthalate to reveal the spots[7]. As judged visually, the 3:1 molar ratio of mannose to galactose found in earlier analyses of ovomucoid[8,9] was unchanged in each fraction.

(*d*) *Sialic acid content*. Using the diphenylamine method[10] with N-acetylneuraminic acid as standard, and assuming that an E_{1cm} of 0.45 at 280 mμ corresponds to a concentration of 1 mg/ml for all fractions, the values for the sialic acid content ranged from 0.9–1.3 % for the early fractions from various runs, while the late fractions gave higher values of 1.8–2.3 %. These results suggested that the basis of the heterogeneity might lie in variations in sialic acid content, those molecules containing more of the acid being held more firmly by the anion exchanger. However, with the "direct Ehrlich" reaction[11], no fraction gave a value greater than 0.5 % sialic acid, which corresponds to appreciably less than one residue per molecular weight of approx. 28,000[1,2]. This matter cannot therefore be regarded as settled.

Heterogeneity of ovomucoid was first indicated by the observation that it exhibits reversible boundary spreading in moving-boundary electrophoresis[12], and at the unusually low ionic strength of 0.01, resolution into components has been observed[2,13]. By zone electrophoresis at an ionic strength of 0.1, however, no signs of such heterogeneity were found.[3] Recently, fractions of ovomucoid have been obtained by elution from carboxymethylcellulose between pH 3.5 and 4.3 (ref. 14).

The work reported here suggests a simple batch procedure for the purification of ovomucoid, which has been successfully applied to 2 g preparation B on a column of 20 g triethylaminoethylammonium-cellulose. Conditions were as described for Fig. 1 except that, instead of elution by gradient, contaminants were washed out with 0.004 *M* sodium phosphate buffer (pH 6.8) containing 0.01 *M* NaCl, and the ovomucoid was then eluted by raising the NaCl concentration to 0.2 *M*. Analysis of this material by gradient elution showed no trace of the first and third peaks given by crude preparations. The similarity in the properties of the early and late fractions described above is ground for believing that the ovomucoid so prepared is substantially free of contamination by unrelated protein.

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A modified method for the purification of erythrocuprein

A copper-containing protein named haemocuprein was isolated from the erythrocytes of different mammals by MANN AND KEILIN[1]. The preparation from ox-blood contained 0.34 % Cu, and there was evidence that the protein was obtained in a highly purified state. The authors also tried to purify a corresponding protein from human erythrocytes and obtained a preparation with a copper content of 0.21 %. A copper protein of human erythrocytes was recently highly purified and characterized[2,3] and it was shown to have the same copper content and probably the same molecular weight as ox-blood haemocuprein. The authors[2] suggested the name erythrocuprein for the human-erythrocyte protein, and they showed that erythrocuprein seems to account for all or nearly all of the copper in the erythrocytes.

The preparation method for erythrocuprein described by MARKOWITZ *et al.*[2] is to a great part identical with that used by MANN AND KEILIN[1]. KIMMEL *et al.*[3] simplified this method by replacing some of the later purification steps by an electrophoretic fractionation. As in the procedure of KIMMEL *et al.*[3], the present method of purification retains steps 1–3 in the method of MARKOWITZ *et al.*[2] but the further purification is achieved by chromatography on the chloride form of DEAE-cellulose[4]. The erythrocuprein prepared in this manner shows a slightly higher copper content than the one obtained previously and appears homogeneous in the ultracentrifuge and in free electrophoresis.

The starting material consists of human blood stored for 1–4 weeks at 4° in acid citrate–dextrose solution. Typical runs were performed with 6 l blood. All buffers and salt solutions were made from reagent-grade chemicals in deionized water.

After removal of the hemoglobin by treatment with the chloroform–ethanol mixture, the remaining protein solution was treated with aq. lead acetate, and the precipitate was extracted with 0.33 *M* phosphate buffer, pH 6.0 (for details see MARKOWITZ *et al.*[2]). The phosphate solution was dialyzed against deionized water at 4° until free from phosphate. To the dialyzed solution, Tris–HCl buffer (pH 7.4) was then added to a final concentration of 0.005 *M*. The column used was jacketed and cooled with tap water (8°). It was packed with DEAE-cellulose (from Distillation Products Industries, Rochester 3, N.Y., U.S.A.) and equilibrated with 0.005 *M* Tris–HCl buffer (pH 7.4). The protein solution was then filtered through the column. Approx. 65 % (varying between 50 and 85 %) of the protein material ran through the column. Erythrocuprein is adsorbed to the column and forms an easily detectable light greenish zone. At the top of the column a yellowish-brown zone is usually formed. After washing the column with 200–300 ml 0.005 *M* Tris–HCl (pH 7.4) the elution is performed by means of a concentration gradient (for details, see the caption to Fig. 1). In the elution pattern (Fig. 1) erythrocuprein was localized through its color. The peak behind the erythrocuprein is due to a yellowish-brown protein. It shows a very high H_2O_2-decomposing activity and seems, at least partly, to consist of catalase.

The pooled fractions of erythrocuprein from the chromatographic experiment was extensively dialyzed against deionized water and lyophilized. Usually several different chromatographic preparations of erythrocuprein were pooled and further purified by rechromatography (column dimensions, 1.3 × 18 cm; mixing chamber,

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Abbreviations: DEAE-, diethylaminoethyl-; Tris, tris(hydroxymethyl)aminomethane.

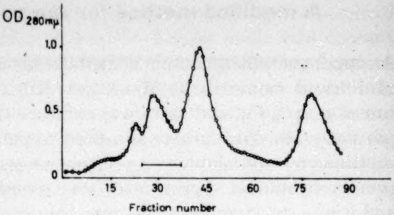

Fig. 1

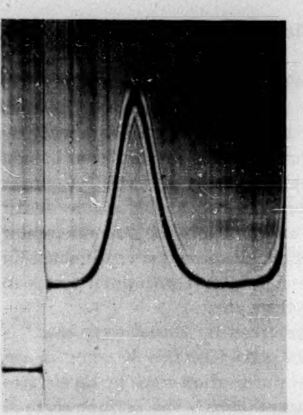

Fig. 2

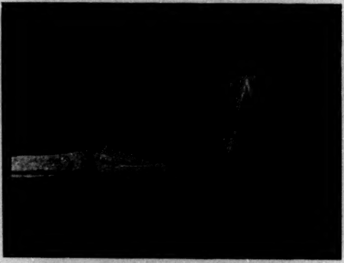

Fig. 3

Fig. 1. Typical elution pattern from DEAE-chromatography for preparation of erythrocuprein. The protein concentration ($OD_{280\ m\mu}$) is expressed as the absorbancy at 280 mμ in a 1-cm cuvette. Column dimensions, 2.0 × 20 cm; concentration gradient (0.005 *M* Tris–HCl, pH 7.4) ⟶ (0.05 *M* Tris–HCl, pH 7.4 + 0.166 *M* NaCl); mixing chamber, 1000 ml; fraction volume, 8.5 ml. Fractions number 25–37 were pooled and purified further by rechromatography.

Fig. 2. Sedimentation pattern of erythrocuprein. Buffer, sodium phosphate, pH 6.5, I 0.05, containing also 0.10 *M* NaCl. The exposure was taken 106 min after reaching 59,780 rev./min.

Fig. 3. Free electrophoresis of erythrocuprein in sodium phosphate buffer, pH 7.0, I 0.10. Ascending pattern after 184 min. Potential gradient, 7.74 V/cm. Protein concentration, 0.5 %. The small peak to the left in the figure is a salt gradient.

400 ml). Erythrocuprein prepared in this way was used for the homogeneity tests (Figs. 2 and 3) and for the determination of elementary composition.

Some experiments with purification by zone electrophoresis in cellulose columns[5,6] (sodium phosphate buffer, pH 6.5, I 0.05) were not successful, since this procedure seems to cause partial denaturation of the protein.

The visible absorption spectrum of the protein was investigated by means of an automatic recording spectrophotometer (Zeiss PRQ 20A). The spectrum was very similar to that given by MARKOWITZ *et al.*[2]. The minimum in the absorption curve is situated at 530 ± 5 mμ, which seems to be the same as obtained previously. The maximum (670 ± 5 mμ), however, differs somewhat from the value given by MARKOWITZ *et al.*[2]. The difference may be due to difficulties in the exact determination of the point of maximum absorption in the broad and flat absorption peak. This is supported by the fact that the erythrocuprein here has been found to give the same electron-spin-resonance spectrum[7] as that prepared by KIMMEL *et al.*[3].

The protein which was to be analyzed was dissolved in deionized water and precipitated with cold absolute ethanol (—30°). The precipitate was washed with absolute ethanol, acetone and ether and air-dried at room temperature. A sample of erythrocuprein prepared in this manner contained 16.88 % N and 1.13 % S (corrected for moisture content in the sample). The copper content was determined by the dithizone method[8] on an aqueous solution of the protein from which the copper was released and the protein precipitated by trichloroacetic acid[9]. The protein concentration was determined by micro Kjeldahl analysis using the nitrogen content of 16.88 %. The copper content was found to be 0.38 (0.376) %.

The elementary composition of erythrocuprein prepared in the described manner seems to be in rather good agreement with the values given previously[2,3]. The molecular weight calculated from the copper content (two copper atoms per protein molecule) is 33,800. This is in good agreement with the value calculated from the amino acid composition[3].

On the basis of the comparisons made above there seems to be no doubts that the protein prepared here is identical to that prepared earlier[2,3] but the present procedure may be somewhat more convenient and it also results in a product showing a higher degree of homogeneity.

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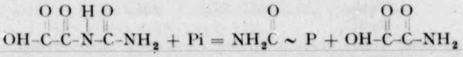
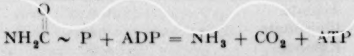
Biochim. Biophys. Acta, 45 (1960) 387–389

Phosphorolysis of carbamyl oxamic acid

Decomposition of the ureido group of carbamyl oxamate by *Streptococcus allantoicus*[1] has been found to be an energy-yielding reaction leading to the formation of ATP. The proposed mechanism for this transformation is as follows:



$$\mathrm{OH{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{H}{|}}{N}{-}\overset{\overset{O}{\|}}{C}{-}NH_2 + Pi = NH_2\overset{\overset{O}{\|}}{C} \sim P + OH{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{O}{\|}}{C}{-}NH_2} \qquad (1)$$



$$\mathrm{NH_2\overset{\overset{O}{\|}}{C} \sim P + ADP = NH_3 + CO_2 + ATP} \qquad (2)$$

Abbreviations: Pi, inorganic phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Tris, tris(hydroxymethyl)aminomethane.

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This communication presents evidence in support of the above reactions. Oxamic transcarbamylase, catalyzing reaction (1), was described previously.[2]

In the experiments to be reported, ATP generation from carbamyl oxamate was coupled with acetate phosphorylation by means of an acetate kinase present in the enzyme preparation. Hydroxylamine was used to trap the acetyl phosphate as it was formed. Under these conditions the equilibrium of reaction(1), which lies toward synthesis of carbamyl oxamate, was displaced since the ATP formed by reaction (2) was utilized in acetate activation. High concentrations of neutral hydroxylamine and acetate did not inhibit the reaction.

ATP was determined under the following conditions: a suitable amount of enzyme was incubated with 20 μmoles carbamyl oxamate, 50 μmoles Tris (pH 7.8), 5 μmoles $MgSO_4$, 20 μmoles potassium phosphate (pH 7.8), 1 μmole ADP, 200 μmoles sodium acetate, 700 μmoles neutral hydroxylamine and water to 1 ml. After 20 min at 30°, the reaction was terminated by the addition of $FeCl_3$ reagent[3] and the acethydroxamic acid formed was determined colorimetrically. Further identification of acethydroxamic acid was achieved chromatographically according to the method of STADTMAN[4].

The enzyme used was an ammonium-sulfate precipitated, dialyzed fraction prepared as described previously[2]. Carbamyl oxamate (Nutritional Biochemical Corp.) and citrulline were determined by the method of ARCHIBALD[5]. Oxamate (Nutritional Biochemical Corp.) was determined by chromatography[2] on Dowex-1-X10 and by an unpublished procedure of SMYTH AND BARKER, kindly supplied by Dr. LEON CAMPBELL. In this method, oxamate is substituted for acetamide in the procedure of LIPMANN AND TUTTLE[3]. Separation of carbamyl oxamate and oxamate was achieved chromatographically using Dowex-1-X10 (acetate form)[2].

Table I shows the requirements for acethydroxamic acid formation from carbamyl oxamate. As seen from the table, the necessary requirements for hydroxamate formation are inorganic phosphate, Mg^{++}, acetate, ADP, hydroxylamine, enzyme and substrate.

The catalytic role of Pi on hydroxamate formation is shown in Fig. 2. 8 μmoles acethydroxamate were formed in 20 min in this reaction. When arsenate replaced Pi no ATP was formed; however, when arsenate was used, carbamyl oxamate rapidly disappeared. Evidence that an arsenolytic cleavage occurs is presented in Fig. 1. In

TABLE I

REQUIREMENTS FOR ATP FORMATION

Conditions: standard assay. Enzyme: 1.0 mg of an ammonium-sulfate precipitated, dialyzed fraction. Incubation: 30° for 20 min.

Constituent omitted	*Acetyl hydroxamate formed (μmoles)*
None	7.6
$MgSO_4$	0.5
ADP	0.2
NH_2OH	0.0
Acetate	0.0
Phosphate	0.4
Enzyme	0.0
Carbamyl oxamate	0.2

this experiment the arsenolysis of carbamyl oxamate was measured by following oxamate formation. Oxamate formation correlated well with carbamyl oxamate disappearance. The phosphorolytic cleavage of carbamyl oxamate, on the other hand, results in the formation of oxamate and carbamyl phosphate. Further evidence for the intermediary role of carbamyl phosphate in this reaction was the finding that carbamyl oxamate is an active carbamyl donor for the synthesis of citrulline from ornithine. This reaction is dependent upon Pi. Furthermore, carbamyl phosphate is rapidly converted to ATP as measured in the standard hydroxylamine assay.

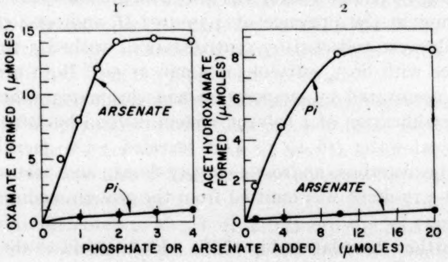

Fig. 1. Arsenolysis of carbamyl oxamate. Each tube contained 19 μmoles carbamyl oxamate, 50 μmoles Tris (pH 7.8), 3 μmoles MgSO$_4$, and potassium arsenate or phosphate as indicated. Enzyme was 1.8 mg ammonium-sulfate precipitated, dialyzed fraction. Total volume was 1 ml; incubation: 30° for 20 min. Fig. 2. ATP formation from carbamyl oxamate. Each tube contained 1 μmole ADP, 20 μmoles carbamyl oxamate, 50 μmoles Tris (pH 7.8), 5 μmoles MgSO$_4$, 200 μmoles sodium acetate, 700 μmoles neutral hydroxylamine, 1.0 mg of enzyme fraction (Fig. 1), and water to 1 ml. Incubation at 30° for 20 min. ATP formation was coupled to the active acetate kinase in this fraction.

The role played by ureido succinate and ureido propionate as energy metabolites in *Zymobacterium oroticum* and *Clostridium uracilium*, respectively, is obscured by the finding that extracts of these organisms hydrolytically cleave the ureido moieties of these acids[7,8]. The significance of these reactions in pyrimidine biosynthesis has been discussed[9,10]. In contrast with these findings, decomposition of the ureido group of carbamyl oxamate by *Streptococcus allantoicus* has been found to be an energy-yielding reaction.

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Formation of testosterone acetate by *Saccharomyces fragilis*

We recently reported that various steroids have an antibiotic effect on the yeast *S. fragilis* during growth[1]. It was further observed that androst-4-en-3,17-dione, such an inhibitor of yeast growth, was converted by intact yeast cells to a less polar compound. In the course of this work, a mutant resistant to steroids was isolated and also found to be capable of performing this transformation. Evidence is presented in this communication that this non-polar steroid is testosterone acetate.

Wild type *S. fragilis* (ATCC 10022) was grown on glucose–salts medium for 48 h at room temperature in the presence of $4.55 \cdot 10^{-4}\,M$ androst-4-en-3,17-dione, as described[1]. The cells were collected by centrifugation, washed 3 times with water, and extracted twice with 80% ethanol for 5 min at 50°. Both ethanolic extracts were combined, concentrated by evaporation and chromatographed on Whatman paper No. 1 in a modification of a solvent system devised by BUSH[2], consisting of cyclohexane–methanol–water (10:10:1). This revealed 3 u.v.-quenching areas with the mobilities of testosterone, androst-4-en-3,17-dione, and testosterone acetate. When androst-4-en-3,17-dione was omitted from the growth medium, none of these three spots was seen.

To establish further the relationship of the added steroid to the two new spots, [4-^{14}C]androst-4-en-3,17-dione, $1.54 \cdot 10^{7}$ counts/min, was added with unlabeled carrier so that the final concentration of steroid in glucose–salts medium was $1.4 \cdot 10^{-5}\,M$. At this concentration of steroid, the growth of wild type and mutant were substantially the same. Cultures of wild type and steroid-resistant mutant were harvested at 50 h and extracted as before. The extracts were chromatographed and the radioactivity located by means of a Forro windowless, gas-flow, strip counter and a Nuclear ratemeter model 1614B coupled to an Esterlene-Angus recording ammeter. Radioactivity was found in three areas corresponding to the added steroid, testosterone, and testosterone acetate, indicating that the latter two were metabolites of the added steroid rather than compounds somehow elicited by it.

Although the mobility of the new non-polar compound corresponded exactly to that of testosterone acetate, it migrated only slightly more slowly than did testosterone propionate. The following studies were therefore undertaken to confirm its identity as the acetate.

The compound could be satisfactorily hydrolyzed to a more polar steroid in 24 h in a saturated solution of ethanolic bicarbonate which suggested that it contained an ester linkage. For convenience, it was usually hydrolyzed in 0.5 N methanolic KOH at 60° for 30 min. When the radioactive material, tentatively identified as testosterone acetate, was eluted from paper, hydrolyzed and re-chromatographed, there was quantitative conversion to an u.v.-quenching compound with the mobility of testosterone. When this latter substance was eluted from the paper, oxidized with chromic acid[3], and re-chromatographed, the radioactivity and u.v. absorption were now in a location corresponding to androst-4-en-3,17-dione, the expected product of testosterone oxidation. This compound was again eluted and enzymically reduced with microsomal Δ^{4}-3-ketosteroid reductase (5α) (ref. 4). The radioactive product had the mobility of the predicted compound, 5α-androstane-3,17-dione.

Thus the steroid moiety of the non-polar compound appeared to be testosterone. To identify the acyl moiety, the original material was hydrolyzed as above, acidified with conc. H_2SO_4, and the volatile products steam distilled. The first 5 ml of distillate were collected, kept alkaline with NH_4OH, concentrated by evaporation and chromatographed in the ascending system of 95 % ethanol and 1 % conc. NH_4OH, described by KENNEDY[5]. The ammonium salt of the steam-volatile material was detected by spraying the chromatogram with bromphenol blue and had the mobility of ammonium acetate.

For further identification of the non-polar steroid, it was again chromatographed on paper together with standard samples of testosterone propionate and testosterone acetate. After development, the three steroids were eluted from the paper, concentrated, and their infrared spectra examined in the solid state by the KBr-pellet method. The spectrum of the unknown corresponded closely with that of the acetate and was clearly different from the propionate spectrum.

It has thus been demonstrated that *Saccharomyces fragilis* can convert androst-4-en-3,17-dione to testosterone acetate. This is a beneficial conversion for the yeast since it is killed by the former steroid while its growth is not affected by the latter[1].

This transformation is also of interest since no steroid-17-esters have previously been isolated from natural sources*.

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* While this report was in preparation, T. E. WEICHSELBAUM AND H. W. MARGRAF[6] presented evidence for a C-21 steroid acetate in human plasma.

X-ray-diffraction analysis of cell walls of nematode-trapping fungi*

The nematode-trapping fungi are a remarkable group of microorganisms capable of capturing living worms by means of specialized hyphal structures formed in response to the presence of their prey[1,2]. Since trapped nematodes struggle violently but seldom escape, the mycelium of these fungi is endowed with great tensile strength. BLANK[3] demonstrated that chitin is the major component of cell walls of dermatophytes and fungi causing systemic infections of higher animals, and it was of interest to determine the chemical nature of cell walls of fungi that trap and parasitize nematode worms. Three organisms were employed: *Arthrobotrys conoides*, *Dactylaria thaumasia*, and *Dactylella ellipsospora*. *A. conoides* and *D. thaumasia* trap nematodes in networks of adhesive hyphal loops, whereas *D. ellipsospora* forms adhesive knobs for the capture of prey. The majority of known species of nematode-trapping hyphomycetes are included in the genera tested.

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The fungi were cultivated in 4-l Fernbach flasks. Each flask contained 500 ml of a broth with the following % composition: glucose, 3.0; yeast extract, 0.3; $NaNO_3$, 0,366; K_2HPO_4, 0.1; $MgSO_4 \cdot 7H_2,O$ 0.05; KCl, 0.05; and $FeSO_4 \cdot 7H_2O$, 0.001. After inoculation flasks were incubated without agitation for 2 weeks at 28°. The pellicle formed was harvested, washed, and mascerated (Servall Omni-Mixer) in a known volume of distilled water. An aliquot of the resulting suspension was used to determine the dry weight of fungus tissue treated by procedures similar to those employed by FULLER AND BARSHAD[4] for identification of the cell-wall constituents of *Rhizidiomyces* species and briefly described below.

To extract everything but cellulose and chitin, the mycelial fragments were boiled in 4.5 % KOH until little or no cytoplasm was discernible within the cell walls when examined microscopically. The KOH was decanted and the fungus tissue washed with boiling distilled water until neutral. The cell walls were then treated with 2 % acetic acid to remove chitosan and subsequently with boiling water to eliminate the acid. To solubilize and thereby separate cellulose from chitin, the preparation was extracted with Schweitzer's reagent for each of two 12-h periods. The insoluble residue was collected, washed with distilled water until neutral, dried, and X-rayed. The Schweitzer's reagent was neutralized with conc. HCl to precipitate mercerized cellulose.

Samples were prepared for X-ray analysis by grinding the dried material with a mortar and pestle and the fraction that passed through a 325 mesh screen was packed in 0.5-mm diameter glass capillaries and mounted in a 2-radian Debye-Scherrer powder camera. Exposures were made for 3 h with Ni-filtered radiation from a copper-

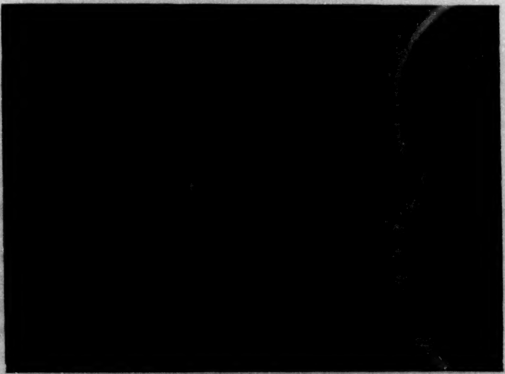

Fig. 1. X-ray diffraction patterns of chitin standard (top), and the cell walls of *A. conoides* (bottom).

target X-ray tube operated at 35 kV and 16 mA. The samples were rotated at a speed of 1.2 rev./min during exposure.

Two or three 1-h extractions in changes of boiling 4.5 % KOH were required to remove cytoplasm and obtain "clean" cell walls. These were insoluble in Schweitzer's reagent and found by X-ray diffraction to be chitin. In no case did neutralization of Schweitzer's reagent cause precipitation of cellulose and no maxima characteristic of cellulose were observed in X-ray-diffraction patterns of cell-wall preparations that were not extracted with Schweitzer's reagent. The X-ray-diffraction maxima for each of the three fungi examined were identical to each other and to a chitin standard prepared by Dr. D. M. REYNOLDS from crab exoskeleton[5]. The most prominent maxima are listed in Table I, and Fig. 1 shows the X-ray-diffraction patterns of the chitin standard and the cell walls of *A. conoides*. The lines at 1.5 and 15 Å are due to a copper–ammonium complex in Schweitzer's reagent.

The data provided unequivocal evidence that a major component of the cell wall of nematode-trapping hyphomycetes is chitin. The chitin content of *A. conoides*, *D. thaumasia*, and *D. ellipsospora* was estimated at 16, 18, and 8 % respectively and was within the range of 2.6 to 26.2 % of dry wt. reported for fungi by BLUMENTHAL AND ROSEMAN[6].

TABLE I

X-RAY DIFFRACTION LINES OF CHITIN

d-Spacing (Å)	*Intensity**
9.5	v.s.
7.0	m.
4.6	v.s.
4.25	w.
4.00	v.w.
3.75	v.w.
3.52	v.w.
3.40	s.
2.77	v.w.
2.58	v.w.
2.30	v.w.

* m, medium; s, strong; v, very; w, weak.

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Vitamin A acid and liver levels of ubiquinone and ubichromenol

Dowling and Wald[1] have recently found that vitamin A acid (the carboxylic acid corresponding to vitamin A, $C_{19}H_{27}CH_2OH$) does not sustain the retinal functions of the vitamin, but they confirmed in more detail earlier reports[2,3] that it was effective in other respects. It maintained normal growth and good outward appearance of animals, and cured the overt signs of vitamin A deficiency. Wodsak[4] has reported that the acid also has activity in the test on cornified vaginal epithelium.

A readily measurable characteristic of the vitamin A-deficient rat is the rise in liver ubiquinone and ubichromenol concentrations[5,6]. (In earlier papers these have been referred to as substances SA and SC respectively.) A preliminary experiment in this laboratory by Redfearn had indicated that animals fed on a vitamin A-deficient diet supplemented with vitamin A acid had liver ubiquinone concentrations in the normal range.

In the present work 24 male weanling rats (weights 31–43 g) were divided into three groups of eight. The first was fed on a diet[6] deficient in vitamin A. Animals in the second group received the same basal diet plus 350 µg vitamin A acid (as the sodium salt) twice weekly; those in the third group had the basal diet supplemented twice weekly with 350 I.U. vitamin A (= 105 µg vitamin A alcohol) in the form of fish liver oil (approx. 0.3 ml). Molecule for molecule, the amount of acid fed was therefore a little over three times that of the vitamin A. Reports[2–4,7] differ as to the biological potency of vitamin A acid, but most assess it as having lower activity than vitamin A itself; the 3-fold larger dose was given to allow for this. The amount was more than adequate for normal development[1], and the increase in weight of the animals fed vitamin A acid was approximately the same as of those given vitamin A. The deficient group grew similarly up to the stage of weight plateau (3–5 weeks); after a time they began to lose weight rapidly as other deficiency signs became severe. They were then killed, together with their companion animals in the other two groups.

The bodies and livers were weighed and the ubiquinone and ubichromenol contents of the livers estimated spectroscopically after saponification and chromatography[6]. Alumina columns were used for ubiquinone[6]; ubichromenol was obtained by further chromatography on magnesium oxide using increasing concentrations of methylal in ether as eluents. The livers of each group were investigated in two batches of three and one of two; the mean results are shown in Table I.

Most of the figures obtained on the separate batches did not differ from the mean shown in Table I by more than 20 %, with the exception of the ubichromenol concentration of deficient animals (up to 35 % difference). There is a clearcut distinction

TABLE I

Group *Diet*	*1* *Deficient*	*2* *Fed vitamin A acid*	*3* *Fed vitamin A*
Mean body weight (g)	67	110	111
Mean liver weight (g)	2.7	5.0	5.1
Mean liver ubiquinone concentration (µmole/g)	0.446	0.150	0.172
Mean liver ubichromenol concentration (µmole/g)	0.061	0.027	$>$0.013*

* See text.

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between the deficient animals on one hand, and those fed either vitamin A or the acid on the other.

An accurate estimate was not possible of the mean liver ubichromenol concentration of animals fed vitamin A. At least 0.013 μmole/g was present, but the true value is higher. Some ubichromenol was lost during the lengthy chromatography needed to separate it from relatively large amounts of vitamin A.

Vitamin A acid is thus effective in maintaining normal not only the body and liver weights but also the ubiquinone levels in rat liver and it prevents a rise in ubichromenol concentration. These facts add to growing evidence that vitamin A acid can fully replace vitamin A alcohol in its "systemic" mode of action.

Vitamin A acid, unlike vitamin A, is not stored in the liver[1,3]. Our results show that the dramatic rise of liver ubiquinone concentration in vitamin A deficiency is part of the deficiency syndrome rather than an indication of the absence of stored vitamin A.

By feeding animals vitamin A acid, it is possible to study minor constituents of liver lipids, unhampered by the presence of vitamin A and its breakdown products which frequently bedevil such work, as with ubichromenol here. Our findings provide some assurance that the lipid picture in the animals will be normal. Furthermore, animals reared on vitamin A acid can rapidly be made avitaminotic, simply by withdrawing it from the diet; this would readily allow the changes in isoprenoid metabolism brought about in vitamin A deficiency[5,6,8,9] to be studied in the mature rat.

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Preliminary Notes

Succinic dehydrogenase of the ergot fungus and mussel

Succinic dehydrogenase has been successfully solubilized and purified from several sources mainly by SINGER, WANG and their co-workers[1,2]. In this communication we wish to report briefly our studies of the solubilization and some properties of succinic dehydrogenase isolated from the mycelium of the ergot fungus, *Claviceps purpurea*, and from the adductor muscle of the bay mussel, *Mytilus edulis*. These organisms are divergent in morphology and in evolutionary development. The dehydrogenase derived from these two sources possesses dissimilarities as expected and also some striking resemblances not only to each other but to the enzyme isolated from other sources.

The *Claviceps* succinic dehydrogenase can be solubilized from its mycelium with great ease, *e.g.* by extraction with acetone, sonic disintegration or merely homogenizing with alumina in a Waring blender for 2 min. In each operation, the activity recovered is more than 75 %. In contrast to the *Claviceps* enzyme, the succinic dehydrogenase of mussel resists solubilization. Sonic disintegration, digestion with snake venom, incubation with wheat germ, microbial or pancreatic lipase, or treatment with lipid solvents either failed to extract or completely inactivated the enzyme. The dehydrogenase is evidently tightly bound to the particulate matter. The particles can be purified through rather drastic treatments. In fact, the purified particles were made by tryptic digestion (1 mg crystalline trypsin to 150 mg protein for 10 min at 35°) and then isolated by differential centrifugation. Only from the acetone powder of the purified particles could the dehydrogenase be brought into solution, with 0.06 *M* tris(hydroxymethyl)aminomethane buffer at pH 8.9. This method resembles the one originally used by SINGER *et al.*[1] for heart-muscle particles. However, butanol, which has been used effectively for the liberation of succinic dehydrogenase from the Keilin–Hartree heart preparation[2], has no solubilizing effect whatsoever on the particles derived from the mussel. The enzyme prepared from *Claviceps* or mussel is in true

TABLE I

THE ACTIVITY OF SUCCINIC DEHYDROGENASE FROM *Claviceps* AND MUSSEL*

	Claviceps	*Mussel*
Mycelium or adductor muscle	7.5	8.2
Particles, purified	—	150
Soluble enzyme, crude	60	270
Soluble enzyme, purified**	300	—

* The activity is expressed in μl O_2 consumed/mg dry weight/h in the assay system with phenazine methosulfate as the electron acceptor.

** The partially purified preparation was made by treatment of the crude soluble extract with protamine sulfate and then by fractionation with ammonium sulfate.

solution and is not sedimented at 140,000 × g. Table I summarizes the two enzymic activities. Succinic dehydrogenase from either *Claviceps* or mussel catalyzes the oxidation of succinate by phenazine methosulfate only. Tetrazolium chloride, methylene blue, ferricyanide and oxygen do not react. The inactivity of ferricyanide is genuine because the assay system which was the same as used by other authors[1,2] responded as usual when the heart preparation was used. This selectivity of electron acceptor is striking; succinic dehydrogenase from all other sources may use ferricyanide with the activity ranging from 10 to 40 % of the rate with phenazine methosulfate.

The soluble succinic dehydrogenase from *Claviceps* is extremely stable in contrast to the enzyme from animal heart or from mussel. The *Claviceps* enzyme, in the partially purified form, can be stored in the frozen condition for 4 weeks without measurable loss in activity. Its half-life in a crude extract is longer than one week at 0°, and about 40 h at 20°. The succinic dehydrogenase activity of the particles from mussel in the form of a suspension is as stable as the Keilin–Hartree preparation. Once the dehydrogenase emerges from the particles it is then very unstable with a half-life less than 24 h at 0°.

The enzyme prepared from either source, reminiscent of the heart enzyme, is not inactivated by the reaction with cyanide performed under the conditions described by KEILIN AND KING[3] when phenazine methosulfate was used as the electron acceptor. However, more rigid tests by reconstitution[4] have not been done.

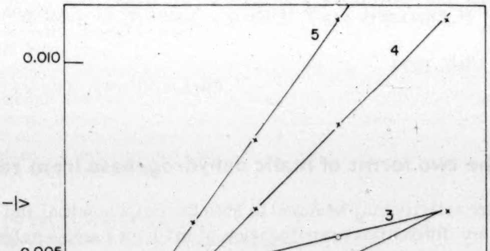

Fig. 1. The inhibition of succinate oxidation catalyzed by the *Claviceps* succinic dehydrogenase. The system contained 0.5 ml 0.3 *M* phosphate buffer, pH 7.7; 0.2 ml 0.15 *M* KCN; 0.2 ml 2 % phenazine methosulfate, 3.2 mg protein; final volume 3.0 ml, temperature 35°. The rate was taken from the initial slope of the oxygen consumption *vs.* time which was linear up to 8 min. Curve 1, with no inhibitor; curve 2, with $5 \cdot 10^{-3}$ *M* fumarate; curve 3, with $3.0 \cdot 10^{-5}$ *M* o-phenanthroline; curve 4, with $5 \cdot 10^{-3}$ *M* pyrophosphate; and curve 5, with $5 \cdot 10^{-4}$ *M* malonate.

The dehydrogenase solubilized from *Claviceps* shows a kinetic behavior similar to that from heart particles. Fumarate, malonate and pyrophosphate competitively inhibits the oxidation of succinate with inhibition constants of $9.3 \cdot 10^{-4}\,M$, $3.0 \cdot 10^{-5}\,M$ and $4.2 \cdot 10^{-4}\,M$, respectively (Fig. 1). *o*-Phenathrolin partially inhibits the oxidation in an apparently non-competitive manner whereas α,α'-dipyridyl even at $3 \cdot 10^{-3}\,M$ does not show inhibition. The Michaelis constant for succinate is $3 \cdot 10^{-3}\,M$, which is somewhat higher than that for the heart enzyme but lower than that for the *Micrococcus* succinic dehydrogenase ($5.3 \cdot 10^{-3}\,M$)[5].

Cultivation of *C. purpurea* was described previously[6]. The adductor muscle was dissected from mussel which was collected from the bays on the Oregon Coast.

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Nature of the two forms of malic dehydrogenase from rat liver

Malic dehydrogenase activity may be found in both the mitochondrial and supernatant fractions obtained by differential centrifugation of rat-liver homogenates[1,2]. Investigation of the nature of the two forms was pertinent to the purification studies of MDH reported earlier from this laboratory[3,4]. The chromatographic and electrophoretic behavior of the two forms (obtained by procedure C, below) is in general agreement with other recent studies[2,5]. The two preparative procedures A and B, outlined below, also yield fractions of MDH which have chromatographic and electrophoretic characteristics similar to either the "mitochondrial" or the "supernatant" forms. However, the experiments to be described in this communication demonstrate that the "supernatant" form can be converted to the "mitochondrial" one on treatment with *n*-butanol.

The three procedures are as follows. *Procedure A.*[3] 1, Homogenization of rat liver with aq. EDTA-NaCl; 2, heat treatment; 3, alcohol fractionation; 4, chromatography on Amberlite XE-64, which yields two fractions. About 30–40 % of the enzyme is not retained and passes through the column along with a large amount of colored proteins

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Abbreviations: MDH, malic dehydrogenase; DEAE-, diethylaminoethyl-; EDTA, ethylenediaminetetraacetic acid.

("red peak" MDH); the second fraction is retained and eluted by an ionic-strength gradient. 5, The fraction eluted from the resin in step 4 is purified by starch-paste electrophoresis. *Procedure B.*[4] 1, Homogenization of rat liver with *n*-butanol–aq. EDTA-NaCl; 2, heat treatment 3, $(NH_4)_2SO_4$ fractionation; 4, chromatography on Amberlite XE-64; 5, chromatography on DEAE-cellulose. *Procedure C.* Rat liver was homogenized in 0.25 *M* sucrose and centrifuged essentially according to SCHNEIDER[6]. The 700–5,000 × *g* (10 min) precipitate and the 20,000 × *g* (60 min) supernatant were designated as the "mitochondrial" and "supernatant" fractions, respectively.

TABLE I

CHROMATOGRAPHIC AND ELECTROPHORETIC BEHAVIOR OF MDH FRACTIONS

Chromatography on Amberlite XE-64 was carried out by gradient, 0.02 *M* → 0.1 *M* sodium citrate, pH 6.2–6.3. Starch-paste electrophoresis was performed in 0.05 *M* sodium citrate, pH 5.4, for about 20 h at 300 V, 4°.

*Fraction of MDH**	*Amberlite XE-64*		*Electrophoresis*	
	% bound	*% not bound*	*% at cathode*	*% at anode*
I. Procedure A				
1. MDH from step 3	70	30	70	30
2. MDH from step 4 (bound to resin)	—	—	100	0
3. MDH from step 4 ("red peak")	0	100	0	100
4. "red peak" MDH, butanol-treated	99	1	—	—
II. Procedure B				
5. MDH from step 3	98	2	70	30
6. MDH from step 4 (bound to resin)			98	2
7. Pure MDH from step 5**			100***	
III. Procedure C				
8. "Mitochondrial" fraction	99	1	98	2
9. "Supernatant" fraction	0	100	10	90
10. "Supernatant", butanol-treated	98	2	40	60
11. "Supernatant", butanol-treated and eluted from Amberlite XE-64			90	10

* See text for description of fractions.
** This fraction is unretarded by DEAE-cellulose.
*** Moving-boundary electrophoresis.

As shown in Table I, the chromatographic and electrophoretic properties of the "red peak" MDH (procedure A) are identical to the "supernatant" form (procedure C). Rechromatographing of the red peak MDH on Amberlite XE-64 several times is without effect. However, if the same fraction is stirred with *n*-butanol for 15 min, dialyzed and then applied to the resin, it is bound and purified over 6-fold. The supernatant MDH (procedure C) also binds to the resin after treatment with *n*-butanol.

In contrast to procedures A and C, every step of procedure B yields over 98 % of the enzyme as a single fraction. That one of the forms is not lost through inactivation during purification by procedure B is supported by the following: 1, the initial extraction step yields at least as much total enzyme as does procedure C, and 20–30 % more than does procedure A; 2, over 95 % of MDH is recovered in every step of procedure B; 3, when the enzyme obtained from step 3 (procedure B) as a single fraction is subjected to starch electrophoresis, it separates into two fractions, one of which moves toward the anode (Table I). Thus, a fraction resembling electrophoretically the supernatant form is still present up to this step. However, if the enzyme from step 3 (procedure B) is chromatographed on Amberlite XE-64, 98 % of the MDH appears as a single peak. The total recovery of MDH is again over 90 %. When eluted from the resin, the enzyme is shown to be electrophoretically homogeneous. Thus, both treatment with butanol and chromatography on Amberlite XE-64 are necessary for the complete conversion of the enzyme to a single form.

The conversion of the "supernatant" form (procedure C) follows the same pattern. Electrophoresis of the butanol-treated "supernatant" shows that 30–40 % has been converted to the "mitochondrial" form. However, the butanol-treated "supernatant" binds to Amberlite XE-64, and the eluted enzyme moves toward the cathode (Table I).

The well known ability of butanol to break up lipid complexes suggests a possible explanation of the results. The "supernatant" form may be a lipoprotein complex of MDH. Treatment with butanol dissociates part of the complex. The remaining complex may be sensitized enough so that the resin can now resolve it completely.

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The isolation and structure of cerebrosides from wheat flour

Our interest in the chemistry and biochemistry of plant lipids led us to a study of the benzene-extractable lipids of wheat flour[1], from which we have now been able to isolate and characterize a mixture of cerebrosides.

Unbleached wheat flour was extracted with benzene. Addition of acetone caused precipitation of an insoluble fraction. The benzene-acetone-soluble material was distributed between *n*-heptane and methanol and the methanol-soluble lipids were purified using a silicic acid column. The fraction eluted with chloroform–methanol (94:6) was rich in cerebrosides, as established by color tests, elementary analyses and infrared spectrum.

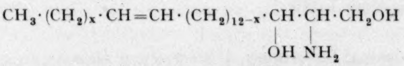
Acid hydrolysis of this material and extraction of the basic components by the method of Carter *et al.*[2] gave a mixture of long-chain bases, which were purified by silicic acid chromatography. The fraction eluted with chloroform–methanol (1:4) was ninhydrin-positive and in the infrared had a pattern very similar to that of brain sphingosine. The maximum at 970 cm^{-1} was observed, indicating the presence of a *trans*-double bond. Periodate oxidation of this material, followed by vapor-phase chromatography of the long-chain aldehydes by the method of SWEELEY AND MOSCATELLI[3], gave the results reported in Table I. On the basis of the retention time of aldehydes from the unknown mixture compared with these of known standards, we were able to identify the presence of following bases: (1) phytosphingosine (peak a); (2) dehydrophytosphingosine* (peak b); (3) dihydrosphingosine (peak c); (4) a new, long-chain base, with isolated double bond (peak d). To this base we have assigned the formula:

TABLE I

THE RETENTION TIME OF ALDEHYDES ISOLATED AFTER PERIODATE OXIDATION OF THE CORRESPONDING BASE*

Base(s)	Peak Retention time (min) of aldehyde				
	a	*b*	*c*	*d*	*e*
1 From wheat-flour cerebrosides	6.6	7.2	8.2	9.3	—
2 From wheat-flour cerebrosides** (hydrogenated aldehydes)	6.6	—	8.2	—	—
3 Dihydrosphingosine	—	—	8.2	—	—
4 Sphingosine	—	—	—	—	13.2
5 Phytosphingosine	6.8	—	—	—	—
6 Dehydrophytosphingosine	—	7.4	—	—	—
7 Mixture of 3, 4, 5, and 6	6.8	7.4	8.5	—	13.2

* Column as described in ref. 3. Temperature, 199°; 12 lb/in².
** Unsaturated aldehydes in mixture of aldehydes, were hydrogenated to fully saturated aldehydes, as described in ref. 3.



$$CH_3\cdot(CH_2)_x\cdot CH{=}CH\cdot(CH_2)_{12-x}\cdot\underset{OH}{CH}\cdot\underset{NH_2}{CH}\cdot CH_2OH$$

This structure was confirmed by the following studies. The crude cerebroside fraction, isolated as described above, was hydrolyzed with 1 *N* NaOH at 37°, fatty acids were removed using Dowex-2 (OH-phase) and Amberlite-MB-3 columns, and the cerebroside fraction was purified by crystallization, yielding a white powder with elementary analysis: C, 66.72; H, 11.09; N, 2.07 %. The infrared spectrum of this sample showed the presence of hydroxyl-, amide- and *trans*-double-bond absorptions. The material was oxidized with sodium periodate in aqueous methanol, followed by $NaBH_4$ reduction and then by mild acidic hydrolysis (0.1 *N* HCl at room temperature). Chloroform extraction of the reaction mixture gave ceramides, free of phytosphingosine and dehydrophytosphingosine-containing material. After purification on silicic acid, a substance with C, 73.92; H, 12.02; N, 2.02 %, and an infrared spectrum typical for

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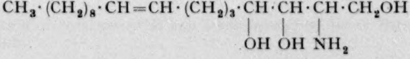
* Isolated from soybean in this laboratory and tentatively assigned the structure



$$CH_3\cdot(CH_2)_8\cdot CH{=}CH\cdot(CH_2)_3\cdot\underset{OH}{CH}\cdot\underset{OH}{CH}\cdot\underset{NH_2}{CH}\cdot CH_2OH$$

ceramide, was obtained. A double bond at 970 cm^{-1} was detected. Periodate consumption of this material was zero. On the assumption that this ceramide was a mixture of dihydrosphingosine and the new ceramide containing the long-chain base, catalytic hydrogenation (in presence of Adams catalyst) was performed. A white powder with m.p. 109–114° was obtained with C, 73.77; H, 12.42; N, 2.25 % (calcd. for hydroxystearoyl-dihydrosphingosine: C, 74.04; H, 12.60; N, 2.40 %).

Alkaline hydrolysis of the reduced ceramide in aqueous dioxane followed by acidification and extraction with chloroform gave a mixture of fatty acids, from which using silicic acid chromatography, α-hydroxystearic acid was isolated as the major component. The melting point (93–94.5°), elementary analysis (C, 72.50; H, 12.15; neutralization equivalent, 296; calcd.: C, 71.94; H, 12.08; neutralization equivalent, 300) and infrared spectrum confirmed this structure, although the presence of other fatty acids was also observed.

From aqueous solution, after adjusting the pH to 12, the long-chain base was extracted with ether. The white powder obtained had the elementary composition: C, 71.81; H, 12,67; N, 4.48 %. The periodate oxidation of this base yielded formic acid and formaldehyde (isolated in form of dimedone derivative in 49 % yield of theory). In addition, the free base was converted to the tribenzoyl-derivative, which after recrystallization had m.p. 146–148.5° (undepressed with authentic tribenzoyl-dihydrosphingosine), infrared spectrum identical with authentic tribenzoyldihydrosphingosine and with elementary analysis: C, 76.29; H, 8.62; N, 2.31 % (calcd.: C, 76.31; H, 8.38, N, 2.28 %).

The sugar component of this cerebroside was identified after acidic hydrolysis as glucose by paper chromatographic technique as well as by the method of ROSENBERG AND CHARGAFF[4].

The discovery in plant sources of dihydrosphingosine and of an isomer of sphingosine represents a novel development in the knowledge of long-chain bases.

Full details of these investigations will be published elsewhere. The authors wish to express their appreciation to the Procter and Gamble Co. for a research grant and for a supply of wheat-flour lipids.

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Identification of the triose phosphate formed in the tryptophan synthetase reaction

In *Escherichia coli* tryptophan synthetase is a two-component enzyme catalyzing the following three reactions[1,2]:

Indole + L-serine $\longrightarrow$ L-tryptophan ([illegible]
Indole-3-glycerol phosphate + L-serine $\longrightarrow$ L-tryptophan + triose phosphate (2)
Indole-3-glycerol phosphate $\leftrightharpoons$ indole + triose phosphate (3)

Reactions (1) and (2) require pyridoxal phosphate as a cofactor. Reaction (2) is responsible for tryptophan synthesis in the normal cell[3]. When the enzyme is presented with indole-3-glycerol phosphate (InGP) or indole and a mixture of triose phosphates in the absence of L-serine, reaction (3) can be shown to occur. In the present experiments, in order to determine whether glyceraldehyde 3-phosphate or dihydroxyacetone phosphate is the triose phosphate concerned in reactions (2) and (3), purified *E. coli* tryptophan synthetase has been coupled with either glyceraldehyde 3-phosphate dehydrogenase* or α-glycerol phosphate dehydrogenase. Reactions were followed spectrophotometrically by noting the change in absorbancy of DPN or DPNH at 340 mμ.

The more acid-stable protein component of *E. coli* tryptophan synthetase, component A, was purified as previously described[1] from the supernatant of a $MnCl_2$-treated extract of a strain (B-8) producing large amounts of this component. Chromatography of a pH-4.0 supernatant on a DEAE-cellulose column resulted in a preparation of component A which was electrophoretically homogeneous[1], free of tryptophan synthetase component B, and nearly free of triose phosphate isomerase. Component B was prepared from a similar $MnCl_2$ supernatant of a strain (A-2) rich in this component. Ammonium sulfate fractionation followed by chromatography on DEAE-cellulose** resulted in a preparation which was free of component A and contained only small amounts of DPNH oxidase and triose phosphate isomerase activity.

Mixtures of components A and B, when added to cuvettes containing InGP, DL-serine and pyridoxal phosphate in addition to the glyceraldehyde 3-phosphate dehydrogenase system[4], rapidly reduced amounts of DPN stoichiometric to the amount of InGP added (Fig. 1). (No absorbancy change was observed when glyceraldehyde 3-phosphate dehydrogenase was omitted.) The same tryptophan synthetase components and substrates, when added to cuvettes containing an α-glycerol phosphate dehydrogenase system[4] caused only a slow oxidation of DPNH unless triose phosphate isomerase was also added (Fig. 2); the amount of DPNH oxidation observed in the absence of added triose phosphate isomerase was that expected from the small amounts of DPNH oxidase and triose phosphate isomerase contaminating the tryptophan synthetase preparations. It can be seen from Figs. 1 and 2 that no reaction was observed when one of the two *E. coli* components was omitted.

When reaction (3) was examined by omitting DL-serine and pyridoxal phosphate from the cuvettes, entirely analogous results were obtained. Again the amount of

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Abbreviations: InGP, indole-3-glycerol phosphate; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; DEAE-, diethylaminoethyl.

* Glyceraldehyde 3-phosphate dehydrogenase, α-glycerolphosphate dehydrogenase, and a mixture of the latter enzyme and triose phosphate isomerase were all products of C. F. Boehringer and Sons, Mannheim.

** A similar purification of component B has been developed by O. SMITH AND C. YANOFSKY (personal communication).

DPN reduced or oxidized was stoichiometric with the InGP added. The rate of reaction (3), however, was about one-tenth that of reaction (2). This finding is in agreement with previous results using different assay techniques and conditions[1]. Similar activity ratios have been reported by YANOFSKY AND RACHMELER[3] using the single component tryptophan synthetase of *Neurospora crassa*.

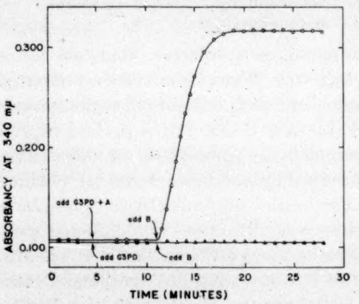

Fig. 1. Cuvettes contained 0.02 *M* NaP_2O_7, buffer, pH 8.5, 0.003 *M* Na_2HAsO_4, 1.5 μmoles DPN, 0.03 % DL-serine, 0.001 % pyridoxal phosphate, and 0.1 μmole InGP. 68 units* of glyceraldehyde 3-phosphate dehydrogenase (G3PD), 11.6 units of component A and 5.6 units of component B were added at times indicated by the arrows.

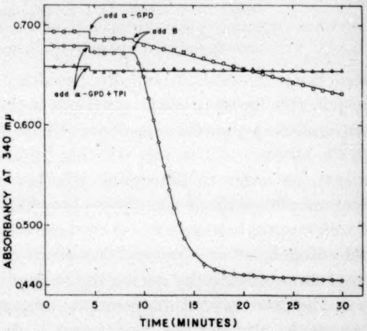

Fig. 2. Cuvettes contained 0.02 *M* NaP_2O_7 buffer, pH 8.5, 0.3 μmole DPNH, 0.3 % DL-serine, 0.001 % pyridoxal phosphate, 0.1 μmole InGP and 11.6 units of component A. 58 units of α-glycerol phosphate dehydrogenase (α-GPD) *or* 68 units of α-glycerol phosphate dehydrogenase plus excess triose phosphate isomerase (TPI), and 5.6 units of component B were added at times indicated by the arrows.

RACHMELER[5] found that when *N. crassa* tryptophan synthetase was presented with both indole and InGP as substrates for conversion to tryptophan, indole was utilized exclusively. In his experiments the fate of the triose phosphate moiety was unknown. When a similar experiment was performed with the *E. coli* enzyme by furnishing indole and InGP in equal concentrations in an experiment such as that of Fig. 1, about a 50 % decrease in the rate of DPN reduction was found. Even increasing the ratio of indole to InGP to 10:1 did not abolish glyceraldehyde 3-phosphate production. These results may indicate that *E. coli* tryptophan synthetase can utilize indole and InGP concurrently in the formation of tryptophan. Additional studies bearing on this problem are in progress.

The present experiments have demonstrated that glyceraldehyde 3-phosphate is the sole product formed from the glycerol phosphate side-chain of InGP during the tryptophan synthetase reaction. Obviously, the coupled reactions illustrated form a rapid and convenient way of assaying the purified enzyme in two of its reactions.

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[*] A unit of enzyme is that amount capable of causing the disappearance of 0.1 μmole of substrate or the appearance of 0.1 μmole of product in 20 min under the conditions of the experiment. Units of components A and B are determined in a 3-fold or greater excess of the other component.

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Structure chimique de la moitié N-terminale du lysozyme de blanc d'oeuf de poule

Au cours de l'étude de la structure chimique du lysozyme de blanc d'oeuf de poule, le pentapeptide N-terminal déjà connu[1]: H–Lys–Val–Phe–Gly–Arg a pu être intégré dans une séquence contenant soixante des quelques 130 acides aminés de la protéine. Huit peptides obtenus par hydrolyse trypsique ("unités trypsiques") du lysozyme réduit par l'acide thioglycolique puis traité par l'acide iodacétique[2] se sont en effet trouvés placés dans cet enchaînement N-terminal compte tenu des résultats acquis dans l'étude des peptides formés au cours de l'hydrolyse chymotrypsique ("unités chymotrypsiques") et contenant des résidus d'acides aminés basiques.

Sur les 8 unités trypsiques, 7 avaient déjà été décrites précédemment[1,3]:

T 1: Lys libre
T 2: Arg libre
T 3: Val–Phe–Gly–Arg
T 4: His–Gly–Leu–Asp–Asp(NH_2)–Tyr–Arg
T 5: Cys–Glu–Leu–Ala–Ala–Ala–Met–Lys
T 6: Phe–Glu–Ser–Asp(NH_2)–Phe–Asp(NH_2)–Glu(NH_2)–Ala–Thr–Thr–Asp(NH_2)–Arg
T 7: Asp–(Gly,Ser,Thr,Thr,Asp)–Asp–Tyr–Gly–Ileu–Leu–(Glu,Ileu,Asp,Ser)–Arg

La structure de la huitième unité:

T 8: Gly–Tyr–(Gly,Ser,Leu)–Asp(NH_2)–Try–Val–Cys–Ala–Ala–Lys

n'a été établie que récemment[4]. On sait de plus que T 2 s'enchaîne à T 5 car il a été possible d'isoler dans l'hydrolysat trypsique le peptide: Cys–Glu–Leu–Ala–Ala–Ala–Met–Lys–Arg.

Quelques unités chymotrypsiques du lysozyme avaient déjà été étudiées en partant de la protéine dénaturée par la chaleur[5]. Cependant parmi ces peptides aucun ne pouvait contenir de demi-résidu de cystine. Cette étude a été reprise en soumettant le lysozyme réduit par l'acide thioglycolique et traité par l'acide iodacétique[3] à l'action de la chymotrypsine (pH 7.5; 3 h; 37°; rapport enzyme/substrat: 1/50; concentration en protéine: 1 %). On sait qu'après réduction chaque résidu de cystine est transformé en deux résidus de S-carboxyméthylcystéine[2,3]; tous les autres acides aminés et même le tryptophane restent inchangés, à l'exception de la méthionine[6]: des peptides contenant à la fois des résidus de cystine et de tryptophane pouvaient ainsi être isolés; c'est en effet la présence de ces deux acides aminés qui rend si difficile l'étude du lysozyme et la différencie de celles de l'insuline, de la ribonucléase et de la protéine du virus de la mosaique du tabac. Les unités chymotrypsiques ont été séparées par chromatographie sur colonne de résine Dowex 50X2 (100 × 4 cm en partant de 500 mg de lysozyme): 24 pics ont été repérés grâce à un dosage à la ninhydrine. Comme certains contenaient plusieurs peptides leur purification a été achevée par chromatographie ou ionophorèse sur papier après dessalification. Parmi les nombreux peptides isolés figurent seuls ici ceux qui ont permis de réunir les unités T 1 à T 8. (R_F dans le solvant: *n*-butanol–acide formique–eau (75, 15, 10, v/v/v); mobilité *m* par rapport à celle de l'arginine = +1 et de l'acide cystéique = −1, à pH 6.5):

C 1: Lys–Val–Phe (pic 20; $m = +0.4$; R_F, 0.35)
C 2: Gly–Arg–Cys–Glu–Leu (pic 16; $m = -0.25$ R_F, 0.12)
C 3: Ala–Ala–Ala–Met–Lys–Arg–His–Gly–Leu–Asp–Asp(NH_2)–Tyr (pic 24; $m = +0.30$; R_F, 0)
C 4: Arg–Gly–Tyr (pic 21; $m = +0.5$; R_F, 0.20)
C 5: Val–Cys–Ala–Ala–Lys–Phe (pic 17; $m = 0$; R_F, 0.27)
C 6: Arg–Asp–(Gly,Ser,Thr,Thr,Asp)–Asp–Tyr (pic 16; $m = -0.23$; R_F, 0)

C 1 réunit T 1 et T 3; C 2 réunit T 3 et T 5; C 3 réunit T 5, T 2 et T 4; C 4 réunit T 4 et T 8; C 5 réunit T 8 et T 6. Enfin T 6 et T 7 avaient déjà été réunis[1] grâce à un peptide obtenu par hydrolyse pepsique du lysozyme et aussi grâce à C 6. Ainsi les unités T 1 à T 8 constituent, une fois réunies, l'enchaînement N-terminal du lysozyme et correspondent à la moitié environ de sa molécule. Dans la formule ci-dessous, les flèches indiquent les liaisons scindées par la chymotrypsine; son action s'est exercée spécifiquement au niveau des groupements carboxyliques des résidus des acides aminés aromatiques et de la leucine, avec une seule exception au niveau d'un résidu d'asparagine.

H–Lys–Val–Phe$\overset{\downarrow}{-}$Gly–Arg–Cys–Glu–Leu$\overset{\downarrow}{-}$Ala–Ala–Ala–Met–Lys–Arg–His–Gly–Leu$\overset{\downarrow}{-}$
Asp–Asp(NH_2)–Tyr$\overset{\downarrow}{-}$Arg–Gly–Tyr$\overset{\downarrow}{-}$(Gly,Ser,Leu)–Asp(NH_2)–Try$\overset{\downarrow}{-}$Val–Cys–Ala–Ala–
Lys–Phe$\overset{\downarrow}{-}$Glu–Ser–Asp(NH_2)–Phe$\overset{\downarrow}{-}$Asp(NH_2)–Glu(NH_2)–Ala–Thr–Thr–Asp(NH_2)$\overset{\downarrow}{-}$
Arg–Asp*–(Gly,Ser,Thr,Thr,Asp*)–Asp–Tyr$\overset{\downarrow}{-}$Gly–Ileu–Leu$\overset{\downarrow}{-}$(Glu*,Ileu,Asp*,Ser)–Arg...

Notons enfin que dans la moitié C-terminale du lysozyme l'enchaînement contenant 34 acides aminés[1] déjà caractérisé vient d'être prolongé: une formule provisoire du lysozyme a pu ainsi être proposée[7].

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* Il n'a pas été déterminé si ces résidus sont amidés ou non.

Cinetique de l'incorporation de la $[^{14}C]$arginine dans differents peptides de l'hemoglobine de lapin

L'étude de l'incorporation du glycocolle[1] et de la phénylalanine[2] dans l'hémoglobine de lapin après incubation de réticulocytes a montré une inégalité de marquage de l'acide aminé dans la globine, après une incubation comprise entre 1 h et 4 h. Ces résultats impliquent une synthèse non instantanée de la molécule protéique avec l'existence possible de peptides intermédiaires libres ou liés à la matrice ribonucléique. Pour étudier avec plus de précision la cinétique de l'incorporation, nous avons hydrolysé la globine préalablement marquée *in vitro* par la $[^{14}C]$arginine après différents temps d'incubation et séparé les peptides formés par la méthode d'Ingram[3]; l'arginine a été isolée des différents peptides et son activité spécifique mesurée.

Les globules rouges de lapin traités à la phénylhydrazine sont incubés selon la technique de Borsook *et al.*[4] modifiée[2] en présence de 20 μC d'arginine par ml de culot globulaire.

L'hémoglobine est préparée selon Roche, Derrien et Moutte[5] et la globine selon Anson et Mirsky[6]. Les fractionnements des chaînes peptidiques α et β sont effectués selon Wilson et Smith[7]; l'hydrolyse trypsique et les "finger prints" sont réalisés selon Ingram[3]. Les autoradiographies effectuées sur film Kodirex durent de 1 à 3 mois.

Les peptides repérés par autoradiographie sont découpés, élués par l'eau, évaporés, repris par HCl 6 *N* distillé 3 fois, hydrolysés en ampoule sous vide. Après lavage, on isole par chromatographie en milieu butanol–acide acétique l'acide aminé qu'on évapore sur lame de verre, on mesure sa radioactivité dans un compteur à gaz, et on le dose selon Rosenberg, Ennor et Morrisson[8]. Chaque expérience est faite sur les peptides de quatre "finger prints", de façon que la quantité d'arginine varie entre 2 et 18 μg.

Les résultats, qui sont résumés dans la Fig. 1 et le Tableau I, montrent une inégalité de marquage variant avec le temps d'incubation; après une incubation de 10 min,

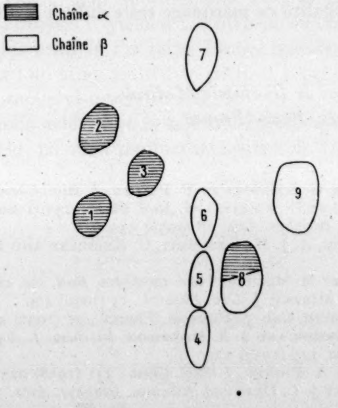

Fig. 1. Nomenclature des peptides.

TABLEAU I

VARIATIONS DE L'ACTIVITÉ SPÉCIFIQUE DE L'ARGININE DES DIFFÉRENTS PEPTIDES DE LA GLOBINE AVEC LA DURÉE D'INCUBATION DES RÉTICULOCYTES

Nature de la chaine		α	α	α		β	β	β	α et β	β
Dénomination des peptides		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Durée d'incubation (min)	10	8	12	11	19	12	30	43	14	34
		13	14	12	23	32	35	55	19	72
	50	120	126	116	180	110	143	136	104	213
		112	106	94	107	110	143	91	107	256
	200	240	225	182	246	203	230	250	218	347
		276		210		254	245	223	288	408

cette inégalité est très accentuée, les activités spécifiques variant dans le rapport 1 à 5, puis elle s'atténue avec le temps, elle reste cependant indiscutable pour au moins 1 peptide après 200 min d'incubation.

Les peptides appartenant à la chaîne α semblent initialement moins marqués que ceux provenant de la chaîne β.

Ces résultats permettent d'exclure une synthèse de la globine selon la conception en "fermeture éclair" avec fermeture simultanée de toutes les liaisons peptidiques et établissent le fait que les différents sites de la globine sont synthétisés à des vitesses différentes.

Il est cependant difficile de déduire de ces résultats l'ordre de formation de ces peptides. En effet le peptide le plus marqué peut aussi bien être celui qui se forme le premier lors de la synthèse de l'hémoglobine, que celui qui se forme le dernier, complétant la synthèse de l'hémoglobine; la complexité du problème a bien été montrée par DALGLIESH[9], les peptides pouvant quitter vraisemblablement en partie la matrice nucléique avant que la molécule protéique soit complètement formée et une seconde molécule pouvant commencer à se synthétiser avant que la précédente soit complète. Si l'on admet que la synthèse de l'hémoglobine dure quelques minutes *in vitro*[10,11] une persistance de l'inégalité de marquage reste difficile à interpréter en l'état actuel de nos connaissances.

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Biochim. Biophys. Acta, 45 (1960) 409–410

Sporidesmolide I, a metabolic product of *Sporidesmium bakeri* Syd.

Current interest in the pasture fungus *Sporidesmium bakeri* Syd. (= *Pithomyces chartarum* (Berk. & Curt.) Ellis) results from its association with a disease of ruminants known as facial eczema[1–4]. One important metabolic product of this fungus[5,6] is probably identical with the "beaker test" substance[7,8] isolated from toxic pastures. This note reports the isolation and probable structure of a major component of the fungal "beaker test" substance. The name "sporidesmolide I" is proposed for this compound.

The chloroform-soluble portion of the crude isolate[6] was recrystallized to constant melting point and specific rotation from chloroform–methanol mixtures. Sporidesmolide I so obtained crystallized from 70% (v/v) acetic acid in fine needles, m.p. 261–263° (corr.), $[\alpha]_{\mathrm{D}}^{17^\circ}$ -217° in chloroform (*c*, 1.5). (Found: C, 62.11, 61.78; H, 9.04, 9.12; O, (detd.), 20.14, 20.01; N, 8.61, 8.96. Calc. for $C_{33}H_{58}O_8N_4$: C, 62.04; H, 9.15; O, 20.04; N, 8.77.). Tests for free amino, hydroxyl and carboxyl groups were negative. Total acid hydrolysis gave α-hydroxy-isovaleric acid, together with valine, leucine and N-methylleucine. Quantitative paper chromatography of the hydrolysate[9] showed that the amino acids were present in the ratio 2:1:1, and permitted the calculation of a minimal molecular weight of 640 ± 10%. Isopiestic measurements in chloroform gave a value of 580, but there was some indication of decomposition[10]. More satisfactory evidence for the molecular weight is being sought.

Sporidesmolide I consumed 2.0 equivalents of alkali for the formula weight of 639. From the resulting solution a mixture of two acids was isolated. One of these was sporidesmolic acid B, $C_{17}H_{32}O_5N_2$, for which the structure L-α-hydroxyisovaleryl-L-valyl-N-methyl-L-leucine has already been proposed[6]. The second, sporidesmolic acid A, was readily separated because of its insolubility in chloroform. It crystallized from aqueous acetic acid as colourless prisms, m.p. 197–199° (corr.), $[\alpha]_{\mathrm{D}}^{17^\circ}$ $+61^\circ$ in glacial acetic acid (*c*, 4). (Found: C, 58.50, 58.43; H, 8.91, 9.08; O, (detd.), 24.23, 24.27; N, 8.67, 8.63. Calc. for $C_{16}H_{30}O_5N_2$: C, 58.16; H, 9.15; O, 24.21; N, 8.48. Eq. wt. (by titration) 332,328; calc.: 330). Sporidesmolic acid A possessed no free amino group. On total acid hydrolysis it yielded L-α-hydroxyisovaleric acid, D-valine and D-leucine, the amino acids being in 1:1 ratio. Partial hydrolysis (conc. HCl, 37°) for 72 h liberated leucine and no other ninhydrin-positive substance. Sporidesmolic acid A is thus L-α-hydroxy-isovaleryl-D-valyl-D-leucine.

The two sporidesmolic acids were isolated in approximately equal amount and in almost theoretical yield; no other product was detected. The evidence is consistent

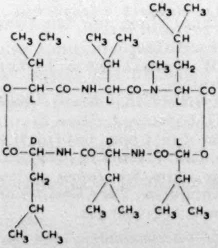

Fig. 1 Cyclic structure for sporidesmolide I.

with a cyclic structure for sporidesmolide I, in which equal numbers of residues of sporidesmolic acids A and B are combined. An 18-membered ring (Fig. 1) is considered most likely, but the question of ring size cannot be settled until unequivocal evidence for the molecular weight is available.

"Strain C" of *S. bakeri* produces in culture some 1–2 % of crude "beaker test" substance[11], of which about two-thirds is sporidesmolide I. Most of the remainder appears to be a homologue, sporidesmolide II, in which the D-valine residue is replaced by one of D-isoleucine. "Strain C" is not a single-spore isolant[12], and its production of two such substances may reflect metabolic differences between constituent true strains.

While the occurrence of D-amino acids in microbial metabolites is to-day almost commonplace, the L-configuration of the α-hydroxy-isovaleric acid residues in sporidesmolide I is noteworthy. The L-isomer does not appear to have been previously reported from natural sources. Since tests have failed to show antibiotic activity in the "beaker test" substance, it seems possible that the antibiotic action of similar compounds[13–16] may be mediated, at least in part, by the D-configuration of certain of their hydroxy acids. The configuration of the β-hydroxytridecanoic acid residue in the related antibiotic esperin[17] is unfortunately at present unknown.

Sporidesmolide I is the most recently investigated member of the class of cyclic hydroxyacyl-aminoacyl compounds for which the generic name "peptolide" was recently proposed[6]. The author's attention has since been drawn to an earlier suggestion[18] that such compounds and their straight-chain analogues be termed "depsipeptides", a name emphasising their analogy to both depsides and peptides. In these circumstances we wish to withdraw the name "peptolide". The chemistry of these compounds has recently been reviewed[19].

It is a pleasure to thank my wife, Mrs. S. L. RUSSELL, for helpful discussions, and Miss M. E. BROWN for valuable assistance.

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PRÉPARATION ET PROPRIÉTÉS DE LA LACTOSIDÉROPHILINE (LACTOTRANSFERRINE) DU LAIT DE FEMME

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(Reçu le 21 juillet 1960)

SUMMARY

Preparation and properties of lactotransferrin of human milk

The authors describe a method of isolating and purifying from human milk a mucoid that has, like plasma siderophilin, the ability to bind iron reversibly. It differs, however, from siderophilin in its physico-chemical and immunological properties. The authors propose to call this specific protein: lactosiderophilin or lactotransferrin.

INTRODUCTION

Nous avons entrepris l'isolement des mucoïdes du lait de Femme d'abord par précipitation fractionnée des protéides par le sulfate d'ammonium, puis par chromatographie sur échangeurs d'ions des précipités obtenus. Ce procédé nous a permis d'isoler du lait de Femme le gynolactomucoïde I (voir ref. 1) et les β-2A-globulines[2]. Au cours des fractionnements, nous avions été frappés par la coloration rose saumon de certains filtrats et précipités; cette coloration s'atténuait lors de l'acidification des solutions et s'intensifiait par neutralisation. Cette observation nous a conduits à l'isolement d'un métalloprotéide: le gynolactomucoïde II, qui possède comme la sidérophiline du plasma sanguin la propriété de fixer réversiblement le fer. Il se distingue cependant de la sidérophiline par ses propriétés physiques, chimiques et immunochimiques. Nous proposons d'appeler ce mucoïde spécifique du lait de Femme: lactosidérophiline ou lactotransferrine***.

Isolement de la lactosidérophiline

3 l de lait de Femme, délipidés par une centrifugation à 0°, sont neutralisés et additionnés d'un volume égal d'une solution aqueuse saturée de sulfate d'ammonium ajustée à pH 7. Le précipité est séparé par filtration et le filtrat, de teinte rose, est amené à pH 3.8. Après un séjour de 2 à 3 jours à 2°, on élimine par filtration un léger précipité. La solution obtenue est alors ajustée à pH 7 et saturée en sulfate d'ammonium. On recueille un précipité rose saumon ("fraction lactosidérophiline")

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[***] Nos résultats ont fait l'objet d'une note préliminaire à l'Académie des Sciences[3] (Séance du 22 février 1960).

qui est redissous dans l'eau distillée. La solution est dialysée et lyophilisée. On obtient environ 5 g d'une poudre rose saumon qui renferme 9.7 g d'oses et 2,14 g d'acide sialique pour 100 g. Les études électrophorétiques et immuno-électrophorétiques de cette préparation révèlent la présence d'au moins 4 composants.

Le précipité obtenu est ensuite soumis à un fractionnement sur colonne d'échangeur de cations faiblement acide (Amberlite XE 64; 3.5 × 60 cm; grain 200–400; forme citrate). La résine est préalablement traitée et purifiée selon le procédé décrit par Hirs[4] et amenée sous la forme citrate selon le mode opératoire de Schmid *et al.*[5]. On la "stabilise" par le passage d'une solution de citrate trisodique 0.05 *M*, amenée à pH 5.2 par addition d'une solution d'acide citrique, jusqu'à ce que les pH des solutions effluente et affluente soient identiques. La solution de protéides (2 g dans 20 ml de citrate trisodique 0.05 *M*), ajustée à pH 5.2, est déposée avec précautions à la surface de la résine et on la laisse s'écouler très lentement (0.5 ml/min). Le développement des protéides est ensuite effectué par passage successif d'une solution de citrate trisodique 0.05 *M* ajustée à pH 5.2, puis d'une solution de citrate trisodique 0.50 *M* ajustée à pH 6.2. La Fig. 1 illustre ce mode opératoire. Le contenu des tubes correspondant à chacun des deux pics majeurs est rassemblé et les protéides sont précipités en saturant les solutions en sulfate d'ammonium à pH 3.8. Les précipités obtenus sont dissous dans l'eau distillée et les solutions sont dialysées, puis lyophilisées.

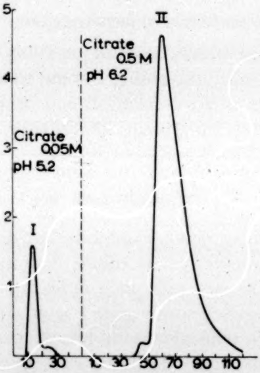


Fig. 1. Chromatographie sur Amberlite XE64 (3.5 × 60 cm; grains 200–400 ; forme citrate) des protéides (2 g) de la "fraction lactosidérophiline" isolée du lait de Femme par la méthode de relargage par le sulfate d'ammonium. En abscisses: nombre de fractions de 10 ml recueillies; en ordonnées: absorbances mesurées à 278 mμ en cuve de 1 cm d'épaisseur.

La solution correspondant au pic I fournit 0,3 g environ d'une poudre blanche qui contient une proportion élevée d'oses (22.5 à 29.25 g d'oses/100 g) et d'acide sialique (5.6 à 5.9 g/100 g) et dont le constituant majeur est le gynolactomucoïde I. Elle ne renferme pas de lactosidérophiline.

La solution rose correspondant au pic II fournit de 0.8 à 1 g d'une substance de couleur rose saumon. L'électrophorèse en gélose (tamponnée à pH 8.2) et l'électrophorèse en gel d'amidon (appareil vertical de Smithies[6]; tampon borate discontinu de Poulik[7]) montrent la parfaite homogénéité de la préparation. L'immuno-électrophorèse en gélose révèle la présence d'un seul arc de précipitation (révélation par un sérum de Lapin antiprotéides du lait de Femme) (Fig. 2).

Propriétés physiques de la lactosidérophiline

Coloration: La lactosidérophiline possède une couleur rose saumon dont la teinte est d'autant plus intense que le chromoprotéide contient plus de fer. Privée du métal, la lactosidérophiline se présente comme une poudre blanche.

Solubilité: La lactosidérophiline est très soluble dans l'eau à pH 7. Elle est précipitée et dénaturée par l'addition de 4 volumes d'éthanol absolu, d'un volume d'une solution aqueuse d'acide trichloracétique à 10 g/100 ml, de 3 volumes d'acide sulfosalicylique 0.2 M ou de 10 volumes d'acide perchlorique 1.8 M. Le sulfate d'ammonium précipite une lactosidérophiline, contenant 2.2 g de fer/1000 g, d'une solution aqueuse à 0.5 g/100 ml, à des concentrations comprises entre 60 et 75 % de la saturation à pH 7 (maximum à 67 %), entre 55 et 75 % de la saturation à pH 5 (maximum à 65 %), et entre 50 à 70 % de la saturation à pH 3.8 (maximum à 50 %).

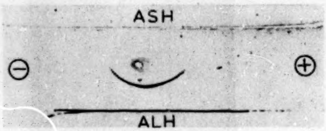
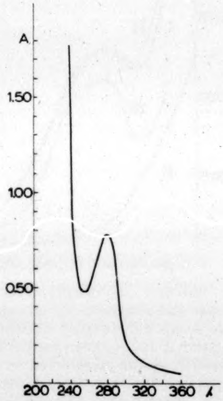


Fig. 2. Immuno-électrophorèse en gélose (tampon véronal de pH 8.2; durée de l'électrophorèse: 2 h) de la lactosidérophiline (à 2.2 g de fer/1000 g). ALH: sérum de Lapin antiprotéides du lait de Femme; ASH: sérum équin antiprotéides sériques humains.

Fig. 3. Spectre d'absorption dans l'u.v. d'une solution à pH 7 de lactosidérophiline saturée en fer (1 g de lactosidérophiline/150 ml d'eau distillée; cuve de 1 cm d'épaisseur). A: absorbances; λ: longueurs d'onde.



Spectres d'absorption: Les solutions neutres de lactosidérophiline saturée en fer présentent, dans l'u.v., un maximum d'absorption à 278.5 mμ et un minimum d'absorption à 255 mμ (Fig. 3). Le coefficient d'extinction, déterminé à 280 mμ et à pH 7, est de:

$$\varepsilon^{10\ \text{mg/ml}}_{1\ \text{cm};\ 280\ \text{m}\mu} = 11.7$$

Le spectre d'absorption de la lumière visible varie avec le pH de la solution et avec le degré de saturation en fer de la lactosidérophiline. La Fig. 4 représente les spectres d'absorption d'une solution aqueuse à pH 7 de lactosidérophiline saturée en fer (courbe I) et de la même solution amenée à pH 1.72 (courbe II). Les solutions neutres de lactosidérophiline saturée en fer possèdent un maximum d'absorption à 452.5 mμ et un minimum d'absorption à 414 mμ. Les coefficients d'extinction déterminés à 452.5 mμ sont les suivants:

$$\varepsilon^{10\ \mathrm{mg/ml}}_{1\ \mathrm{cm};\ 452.5\ \mathrm{m}\mu} = 0.500\ (\pm\ 0.01)$$

$$\varepsilon^{1\ \mathrm{mg\ fer/ml}}_{1\ \mathrm{cm};\ 452.5\ \mathrm{m}\mu} = 0.0144\ (\pm\ 0.0004)$$

Constantes de sédimentation et de diffusion: Les constantes de sédimentation et de diffusion ont été déterminées sur des solutions en tampon phosphate $M/15$ à pH 7.35 d'une préparation de lactosidérophiline renfermant 0.22 % de fer.

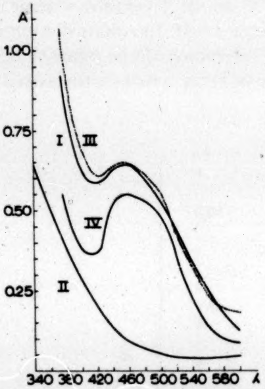


Fig. 4. Spectres d'absorption de la lumière visible par des solutions de lactosidérophiline (cuve de 2 cm d'épaisseur). Courbe I: spectre d'absorption d'une solution neutre à 0.1 g/15 ml de lactosidérophiline saturée en fer. Courbe II: spectre d'absorption de la même solution amenée à pH 1.72. Courbe III: spectre d'absorption de la solution acide précédente ramenée à la neutralité. Courbe IV: spectre de la sidérophiline (d'après FIALA[8]). A: absorbances; λ: longueurs d'onde en mμ.

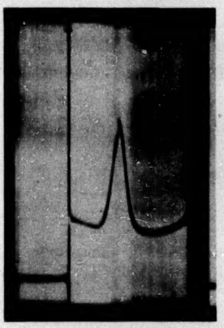


Fig. 5. Diagramme de sédimentation d'une solution de 1 g de lactosidérophiline (2.2 g de fer/1000 g) dans 100 ml de tampon phosphate $M/15$ de pH 7.35 après 50 min de centrifugation (ultracentrifugeuse Spinco modèle E) à la vitesse de 59 780 tours/min.

L'étude des diagrammes d'ultracentrifugation montre l'homogénéité de la préparation (Fig. 5). La mesure de la constante de sédimentation a été effectuée à 20° à l'aide d'une ultracentrifugeuse Spinco modèle E dans une cellule de 12 mm d'épaisseur. Les résultats sont rassemblés dans le Tableau I.

La constante de diffusion a été mesurée à 20° par la méthode de diffusion libre. Elle possède une valeur de 4.6.

TABLEAU I

Concentration (en grammes/100 ml)	*Δn**	*S_{20w}*
1	$1.79 \cdot 10^{-3}$	4.64
0.5	$0.89 \cdot 10^{-3}$	4.65
—	$0.50 \cdot 10^{-3}$	4.7

* Différence entre les indices de réfraction du tampon et de la solution à la concentration indiquée.

Poids moléculaire: Le poids moléculaire de la lactosidérophiline, calculé à partir des constantes de sédimentation et de diffusion et du volume partiel spécifique (0.735), est de 95,000. Cette valeur est en accord avec le résultat apporté par l'étude de la diffusion de la lumière qui permet d'attribuer au poids moléculaire une valeur du même ordre de grandeur: 89,000 ($\pm$ 3,000).

Mobilité électrophorétique: L'électrophorèse sur papier et l'électrophorèse en gélose effectuées à pH 8.9 (tampon véronal) confèrent à la lactosidérophiline saturée en fer une vitesse de β1-globuline. Sa mobilité électrophorétique en veine liquide (Appareil *Aminco*), dans le tampon véronal de pH 8.6 ($\mu = 0.1$), est de $-3.45 \cdot 10^{-5}$ cm^2/V/sec.

Propriétés chimiques de la lactosidérophiline

1. La lactosidérophiline est un métalloprotéide: La teneur en fer a été déterminée par la méthode à l'α,α'-dipyridyle. Elle varie dans de larges proportions suivant les échantillons:

No des échantillons de la lactosidérophiline	*Teneur fer (en g/1000 g de la lactosidérophiline)*
1	0.480
2	1.196
3	0.640
4	2.200

2. La lactosidérophiline fixe réversiblement le fer: Les variations, en fonction du pH, de l'intensité de la coloration des filtrats provenant du fractionnement du lait de Femme par le sulfate d'ammonium, d'une part, la variabilité de la teneur en fer des différentes préparations de lactosidérophiline, d'autre part, nous ont incités à rechercher une éventuelle propriété de fixation réversible du fer par le composé que nous avions isolé.

Fixation du fer: L'application des différentes méthodes décrites à propos de la sidérophiline nous a permis de démontrer que la lactosidérophiline pouvait fixer, *in vitro*, 6 atomes de fer par molécule.

Nous avons mesuré la capacité de fixation du fer par la lactosidérophiline à l'aide de la méthode colorimétrique décrite par SCHADE ET CAROLINE[9], qui est fondée sur l'intensification de la coloration des solutions de sidérophiline par l'addition d'un sel de fer. On ajoute progressivement une solution ferrique à une solution du protéide jusqu'à ce que cesse l'accroissement de l'absorption à 452.5 mμ. On déduit la capacité de fixation du métal par la sidérophiline de la teneur initiale en fer et de la quantité de fer ajoutée à la solution. Ce mode opératoire est parfaitement applicable à la lactosidérophiline.

On introduit 7 ml d'une solution, ajustée à pH 7, de 100 mg de lactosidérophiline dans 15 ml d'eau bidistillée. L'absorbance est déterminée à 452.5 mμ. On ajoute 10 μl d'une solution contenant 5 μg de fer dans 10 μl* et l'on mesure l'absorbance de la solution 5 min après chaque addition. On continue ainsi jusqu'à ce que plusieurs additions successives ne modifient plus l'absorbance. On obtient, de cette manière, des courbes caractéristiques dont l'une est reproduite dans la Fig. 6. La capacité de fixation du fer par la lactosidérophiline, exprimée en grammes/1000 g de protéide, est donnée par la formule suivante:

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* La composition de la solution ferrique est la suivante: protosulfate de fer et d'ammonium (sel de Mohr): 875 mg; HCl 6 *N*: 0.5 ml; eau bidistillée q.s.p. 250 ml.

$$\frac{(p + p')}{P}$$

p: quantité de fer, en μg, contenu dans 7 ml de solution de lactosidérophiline; p': quantité de fer ajoutée, en μg; P: quantité de lactosidérophiline dans 7 ml de solution, en mg.

La lactosidérophiline saturée en fer contient 3.47 g (± 0.13) de ce métal/1000 g, soit 6 atomes de fer par molécule.

Réversibilité de la réaction de fixation du fer: L'abaissement du pH par l'addition d'acide chlorhydrique provoque une diminution progressive de la coloration des solutions de lactosidérophiline "native" (Fig. 7). Les solutions prennent une teinte jaune à pH 2 et sont pratiquement décolorées à pH 1.75. La dissociation du complexe fer–lactosidérophiline est complète pour cette dernière valeur de pH et le fer peut être totalement éliminé par dialyse: on obtient alors, après lyophilisation de la solution dialysée, un protéide incolore.

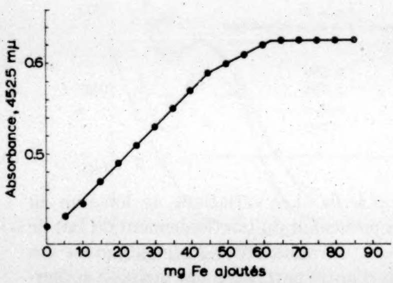

Fig. 6. Courbe de saturation en fer de 7 ml d'une solution neutre de 100 mg de lactosidérophiline renfermant 2.2 μg de fer/mg dans 15 ml d'eau distillée. En ordonnées: absorbances de la solution (mesurées à 452.5 mμ; cuve de 2 cm d'épaisseur).

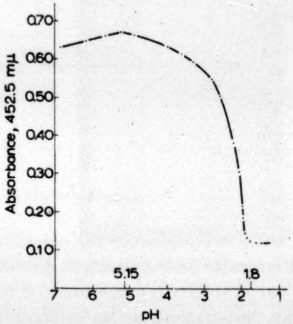

Fig. 7. Courbe de dissociation du complexe fer–lactosidérophiline par l'abaissement du pH d'une solution aqueuse renfermant 100 mg de lactosidérophiline saturée en fer/15 ml d'eau distillée. En ordonnées: absorbances de la solution (mesurées à 452.5 mμ; cuve de 2 cm d'épaisseur).

La dissociation est réversible, puisque le retour à un pH neutre par l'addition de soude à des solutions de lactosidérophiline préalablement décolorées par l'addition d'acide chlorhydrique fait réapparaître la coloration rose avec son intensité initiale (courbe III de la Fig. 4).

3. La lactosidérophiline est un mucoide: La lactosidérophiline se révèle intensément, sur les électrophorégrammes sur papier ou en gélose, par la réaction à l'acide periodique–fuchsine de Schiff. Cette observation, ainsi que les analogies avec la sidérophiline, nous ont amenés à caractériser et à doser les constituants glucidiques de la lactosidérophiline: les oses* par la méthode à l'orcinol de Tillmans et Philippi[11], modifiée par RIMINGTON[12]; les osamines par la méthode de ELSON ET MORGAN[13], modifiée par BELCHER *et al.*[14]; les acides sialiques par la méthode à la diphénylamine de NIAZI ET STATE[15], modifiée par WERNER ET ODIN[16]. Nous avons rassemblé nos résultats dans le Tableau II. Selon la nomenclature de MEYER[17], la lactosidérophiline est donc un mucoïde.

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* L'application de la méthode de chromatographie des oses constituant les glycoprotéides[10] nous a permis de caractériser le galactose, le mannose et le fucose.

DISCUSSION

Nous avons donc isolé du lait de Femme un mucoïde de coloration rose saumon contenant, lorsqu'il est saturé, 3.5 g de fer/1000 g. Il est différent des chromo- et métalloprotéides caractérisés jusqu'à présent dans le lait de Femme.

En effet, la lactosidérophiline ne possède ni les propriétés physico-chimiques, ni l'activité enzymatique de la lactoperoxydase, chromoprotéide porphyrinique contenant 1 atome de fer par molécule d'enzyme[18, 19]. Elle est différente des ferrilactines de BLOCK *et al.*[20–22], qui sont des combinaisons ferriques réversibles de diverses protéines du lait, comme l'α-lactalbumine et la β-lactoglobuline: les ferrilactines sont capables, en effet, de fixer des quantités élevées de fer (jusqu'à 34.2 g de fer/100 g de ferrilactines) et leur coloration varie du blanc au jaune et au brun foncé pour des proportions de fer, respectivement, de 3 à 4 g, 6 à 15 g et 18 à 34.2 g/100 g de ferrilactines.

La lactosidérophiline, dont nous décrivons les propriétés, paraît identique à la métalloprotéine ("*iron-containing red protein*") caractérisée par JOHANSSON[23] au cours de séparations chromatographiques sur des colonnes de phosphate de calcium de la lactalbumine du lactosérum humain, et récemment isolée dans des conditions analogues par le même auteur[24].

La lactosidérophiline et la sidérophiline plasmatique[25, 26] possèdent quelques caractères communs comme la coloration rose saumon et la capacité de fixer réversiblement le fer. Nous avons pu, cependant, faire la discrimination entre la lactosidérophiline et la sidérophiline grâce aux caractères distinctifs suivants:

1. Certaines propriétés physico-chimiques des deux protéides sont différentes. Le Tableau II est à cet égard très démonstratif. On remarque, en particulier: (a) que la lactosidérophiline contient une proportion de glucides plus élevée et fixe une quantité de fer supérieure; (b) que le complexe fer–lactosidérophiline est plus stable en milieu acide que le complexe fer–sidérophiline et se dissocie à un pH inférieur.
2. La lactosidérophiline possède une vitesse de migration électrophorétique en gel d'amidon inférieure à celle des sidérophilines.
3. La lactosidérophiline est immunologiquement différente de la sidérophiline; elle n'est pas révélée, en immuno-électrophorèse en gélose, par un sérum équin antiprotéides sériques humains (Fig. 2). En outre, l'épuisement de cet anti-sérum par la lactosidérophiline ne fait pas disparaître, des diagrammes d'immuno-électrophorèse de sérums humains, l'arc correspondant à la sidérophiline.

L'importance biologique de la lactosidérophiline est évidente. Ce métalloprotéide représente, en effet, la forme de "transport" du fer dans le lait de Femme. Les premiers résultats que nous a apportés l'application d'une méthode de dosage de la lactosidérophiline du lait de Femme, fondée sur le principe de la méthode de dosage de la sidérophiline plasmatique de SCHADE ET CAROLINE[9], montre que le lait de Femme contient, par litre, 1.5 g de lactosidérophiline en moyenne, soit environ 0.75 à 3 mg de fer suivant le degré de saturation en fer du métalloprotéide. Ces nombres sont en accord avec les résultats des dosages de fer effectués dans le lait de Femme par divers

TABLEAU II

PROPRIÉTÉS PHYSIQUES ET CHIMIQUES DE LA LACTOSIDÉROPHILINE ET DE LA SIDÉROPHILINE

	Lactosidérophiline	*Sidérophiline*
λ_{max}	452.5 mμ	460 mμ
$\varepsilon^{10\ mg/ml}_{1\ cm;\ 280\ m\mu}$	11.7	11.2
$\varepsilon^{10\ mg/ml}_{1\ cm;\ 452.5\ m\mu}$	0.500 (± 0.01)	—
$\varepsilon^{1\ \mu g\ fer/ml}_{1\ cm;\ 452.5\ m\mu}$	0.0144 (± 0.0004)	0.017
Constante de sédimentation	4.8 (± 0.1)	5–5.3
Constante de diffusion	4.6	—
Poids moléculaire		
déterminé par la constante de sédimentation	95,000	90,000
déterminé par la diffusion de la lumière	89,000	—
Mobilité électrophorétique en tampon véronal (μ, 0.1; pH, 8.6)	$-3.45 \cdot 10^{-5}$ cm^2/V/sec	$-3.1 \cdot 10^{-5}$ cm^2/V/sec
Capacité de fixation du fer (à pH 7–7.5)		
en μg de fer/mg de protéine	3.47 (± 0.13)	1.25
en atomes de fer/molécule de protéine	6	2
pH de dissociation totale du complexe fer protéine	1.75	4
Azote	14.84 %	15.4 %
Oses (galactose, mannose, fucose)	3.90 %	1.80 %
Osamines	2.40 %	1.30 %
Acides sialique	0.87 %	0.90 %
Oses/acide sialique	4.48	2

auteurs (voir, en particulier, la revue générale de DREYFUS ET SCHAPIRA[27], p. 177 et 178). On peut, d'autre part, penser que les propriétés ferriprives de la lactosidérophiline entraînent, comme dans le cas de la sidérophiline[9], une activité antibiotique vis-à-vis de certains germes pathogènes pour le Nourrisson.

CONCLUSIONS

Le lait de Femme contient un mucoïde de coloration rose saumon qui est capable de fixer réversiblement 6 atomes de fer par molécule. Il ne s'agit cependant pas de la sidérophiline, dont il se distingue par ses propriétés physico-chimiques et par ses caractères immunologiques. Il est, en outre, différent des chromoprotéides et des métalloprotéides isolés jusqu'à présent du lait de Femme. Nous proposons d'appeler ce protéide spécifique: lactosidérophiline ou lactotransferrine.

ADDENDUM À LA CORRECTION

GROVES[28] vient de décrire un procédé d'isolement d'une protéine colorée ("red protein") du lait de Vache sur des colonnes de DEAE-cellulose. Il s'agit d'un glycoprotéide (4.3 à 4.73 g d'oses par 100 g; 2.12 à 2.28 g d'osamines par 100 g; 0.3 g d'acide sialique par 100 g) capable de fixer réversiblement 2 atomes de fer par molécule. Ses propriétés physiques sont les suivantes: $s_{20,w} = 5.55$; $D_{20,w} = 5.75 \cdot 10^{-7}$; P.M. = 86,100; vitesse de migration électrophorétique à pH 9.85: -2.18 à $-2.31 \cdot 10^{-5}$ cm^2/V/sec; pHi: 7,8.

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RÉSUMÉ

Les auteurs décrivent un procédé d'isolement et de purification, à partir du lait de Femme, d'un mucoïde qui possède, comme la sidérophiline plasmatique, la propriété de fixer réversiblement le fer. Il se distingue cependant de la sidérophiline par ses propriétés physico-chimiques et immunologiques. Les auteurs proposent d'appeler ce protéide spécifique: lactosidérophiline ou lactotransferrine.

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DIFFERENCES IN THE NATURE OF NITROGEN PRECIPITATED BY VARIOUS METHODS FROM WHEAT LEAF EXTRACTS

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SUMMARY

1. Four methods of precipitating proteins from leaf extracts were compared.

2. During slow heat coagulation, much nucleic acid is lost, probably from the activity of leaf RNase during heating.

3. After rapid heat coagulation some nucleic acid remains uncoagulated.

4. TCA and perchloric acid behave similarly and precipitate N which consists of protein N as well as nucleic acid N.

5. Uranyl TCA precipitates some TCA soluble polynucleotides and also renders some lipoprotein–polynucleotide complex insoluble in fat solvents.

INTRODUCTION

ROUELLE[1] first demonstrated the presence of proteins in leaves by separating them from a juice of hemlock by heat coagulation, but systematic work on leaf proteins began about 1920 when OSBORNE AND WAKEMAN[2] and CHIBNALL AND SCHRYVER[3] separated crude protein preparations from extracts of spinach and cabbage leaves, respectively. For the next 20 years flocculation by heat, ethanol or by acids like HCl and acetic acid was commonly used to separate proteins from leaf extracts, but of various precipitating reagents tried later, TCA was preferred and heat coagulation and TCA precipitation are now most used.

CHIBNALL AND GROVER[4] used HCl to adjust the pH of leaf extracts and showed that leaf proteins are least soluble between pH 4.0 and 5.0, although they are again precipitated when the pH is taken below 2.5. Their observations have been confirmed with many species of plants. When acids like HCl and acetic acid are used to precipitate leaf proteins, the pH of extracts has to be adjusted accurately, whereas this is not necessary with TCA. Proteins are precipitated from leaf extracts within the wide range of 1–15 % (w/v) final TCA concentration.

The proteins precipitated from leaf extracts by different methods are not necessarily the same. SLADE AND BIRKINSHAW[5] and PIRIE (unpublished) found that precipitates of some leaf extracts by TCA contained 10–20 % more nitrogen than did heat coagula of similar extracts. I have also observed 6–15 % more N in TCA precipitates from leaf extracts of several species of plants than in heat coagula. The

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Abbreviations: TCA, trichloroacetic acid; NA-P, nucleic acid-Phosphorus; NP, nucleoprotein; RNase, ribonuclease.

observations do not suggest that age of leaves affects this difference, but the point has not been studied systematically.

As the different methods of precipitating proteins from leaf extracts seem not to have been studied in detail, the difference in the nature of N precipitated from leaf extracts of some species of plants was studied by four methods, viz. heat coagulation and precipitation by TCA, perchloric acid and a TCA reagent containing uranyl ions. The last two reagents were chosen for study because NEUBERG AND STRAUSS[6] suggested that perchloric acid was a better reagent than TCA and because a Uranyl TCA reagent[7,8] was preferred for studies on nucleic acid and nucleoprotein.

MATERIALS AND METHODS

Leaves (about 200 g) from field-grown wheat (*Triticum vulgare*) were minced in a domestic meat mincer and the sap was squeezed out by hand through a cloth. The residue was reminced and again squeezed. The two lots of sap were mixed and either used at once or stored at 4°. The mixture (extract) from leaves was diluted with 3 volumes of water for all experiments.

Fractionation of extract

The diluted extract was fractionated by differential centrifugation in a cold room. It was first centrifuged at 1,000 × *g* for 7 min and the supernatant fluid was re-centrifuged at 10,000 × *g* for 30 min. These values for centrifugation were chosen because previous work on tobacco leaf extracts made with buffered sucrose[9] showed that starch and intact chloroplasts are sedimented at 1,000 × *g* and chloroplast fragments and mitochondria at 10,000 × *g* leaving an almost clear supernatant fluid.

The sediments from 1,000 × *g* and 10,000 × *g* suspended in water (final volume 20 ml) are referred to as fractions I and II and the supernatant fluid as fraction III.

Precipitation methods

2.0-ml samples of a diluted extract or its fractions were used for precipitation of proteins. The precipitates and the coagulum were sedimented at 1,500 × *g* (10 min), washed with an appropriate medium and again centrifuged at 1,500 × *g* (10 min).

The samples were heat coagulated by leaving them in a boiling water-bath for 5 min, then they were rapidly cooled, centrifuged and washed with water. The precipitation with acid reagents was done by adding to the samples (a) 1.0 ml of 15 % (w/v) TCA, (b) 0.5 ml of 1 *N* $HClO_4$ and (c) 2.0 ml of a Uranyl TCA reagent containing 0.5 % (w/v) uranyl nitrate in 2.5 % (w/v) TCA solution. The precipitated samples were left at 4° for about 1 h and then centrifuged and washed respectively with (a) 5 % TCA, (b) 0.2 *N* $HClO_4$ and (c) the Uranyl TCA reagent diluted with an equal volume of water.

Fractionation of phosphorus compounds of extract

The method, HOLDEN[10] described for fractionating phosphorus compounds of leaf tissues was used with certain modifications.

After precipitation of the extract, the supernatant fluid and the fluid used for washing were pooled, diluted to a suitable volume with 2 *N* H_2SO_4 and a part was taken to estimate total P which is referred to as "soluble P".

Before proceeding with ethanol–ether extraction, the heat coagulum was extracted with 2.0 ml of cold 0.2 N $HClO_4$ and the whole of this extract was used to estimate what will be called "acid soluble P". This cold acid treatment facilitates complete extraction of phospholipids with ethanol–ether[11] and also eliminates interference by non-nucleic acid contaminants when total nucleic acid-phosphorus is estimated spectrophotometrically[12].

Ethanol–ether extraction was done with four successive 2.5-ml lots of ethanol–ether (3:1, v/v), which were pooled and the whole used to estimate total P, referred to as "ethanol–ether soluble P".

This was followed by a single step extraction of total nucleic acid by boiling the residue with 5.0 ml of 1 N $HClO_4$ in a water-bath for 20 min[12]. After separating the insoluble residue by centrifugation, it was washed with 5.0 ml of 1 N $HClO_4$ and again centrifuged. The extract and wash were pooled and used for spectrophotometric estimation of nucleic acid-phosphorus, referred to as "total NA-P".

Chemical estimations

Protein N was determined by a micro-kjeldahl method.

Total P was estimated by the method of HOLDEN AND PIRIE[13] in the precipitates obtained from the extract by various methods. Similarly it was estimated in all the fractions of phosphorus compounds of the extract, except the nucleic acid fraction in which NA-P was measured spectrophotometrically by the method of SPIRIN[12].

RESULTS

Precipitates produced from the extract of wheat leaves by TCA contained 10–15 % more N than did precipitates produced by heat coagulation. The extract was fractionated by differential centrifugation to find which fraction contained most of the "extra N" precipitated by TCA. The fractions and samples of original extract were incubated at 37° for 2 h when their N content was measured and compared with that of unincubated samples and Table I gives the results of one such experiment.

TCA precipitated more N than did heat coagulation from all the fractions but the difference was greatest with fraction III. Less N was precipitated by both techniques from each fraction after incubation than before, but the dimunition was greater with the TCA precipitation than with heat coagulation. Hence the two forms of precipitation gave precipitates that differed less after incubation than before. This suggests that heat coagulation promotes an enzyme action that proceeds slowly during incubation. The two most obvious possibilities are nuclease action and protease action.

PIRIE[14] isolated a nucleoprotein from the supernatant fluid of a tobacco leaf extract and as most of the "extra N" of a TCA precipitate of the wheat-leaf extract also occurs in a similar supernatant fluid, so the total nucleic acid in the two types of precipitate was studied. Table II shows the total NA-P in the fresh and incubated samples both of the unfractionated extract and its fractions.

TCA precipitates contained more NA-P than heat coagula and the difference was again greatest with fraction III. After incubation of the samples, less NA-P was precipitated by both techniques and again the loss was greater in the precipitates produced by TCA.

TABLE I

N-CONTENT OF HEAT AND TCA PRECIPITATES OF WHEAT LEAF EXTRACT AND ITS FRACTIONS

N was determined in a heat coagulum and a TCA precipitate of 2.0-ml samples of wheat leaf extract, diluted four fold, and its fractions and also in similar precipitates of samples incubated at 37°. The values given in the table have been calculated for undiluted extract.

Sample	*Period of incubation at 37° (h)*	*N content of*		*Extra N in TCA precipitate (%)*	*Loss of N during incubation*	
		Heat coagulum (g/l)	*TCA precipitate (g/l)*		*Heat coagulable (%)*	*TCA precipitable (%)*
Unfractionated extract	0	2.00	2.25	11	—	—
	2	1.75	1.83	4	9	19
Fraction I	0	0.29	0.32	8	—	—
	2	0.26	0.27	4	11	15
Fraction II	0	0.6	0.65	8	—	—
	2	0.54	0.56	4	11	14
Fraction III	0	1.12	1.29	15	—	—
	2	1.02	1.1	7	9	15

TABLE II

TOTAL NUCLEIC ACID PHOSPHORUS CONTENT OF HEAT AND TCA PRECIPITATES OF WHEAT LEAF EXTRACT AND ITS FRACTIONS

Total NA-P was measured in a heat coagulum and a TCA precipitate of 2.0-ml samples of wheat leaf extract, diluted four fold, and its fractions and also in similar precipitates of samples incubated at 37°. The values given in the table have been calculated for undiluted extract.

	Period of incubation at 37° (h)	*NA-P content of*		*Extra NA-P in TCA precipitate (%)*	*Loss of NA-P during incubation*	
		Heat coagulum (g/l)	*TCA precipitate (g/l)*		*Heat coagulable (%)*	*TCA precipitable (%)*
Unfractionated extract	0	0.037	0.148	300	—	—
	2	0.015	0.018	20	60	88
Fraction I	0	0.01	0.017	70	—	—
	2	0.006	0.0065	8	40	62
Fraction II	0	0.0045	0.012	166	—	—
	2	0.004	0.0045	12	11	62
Fraction III	0	0.0225	0.12	433	—	—
	2	0.0095	0.0105	10	53	91

A comparison of Tables I and II shows that the loss of 0.42 g of N/l during incubation is accompanied by a loss of 0.13 g of nucleic acid-P. Taking N:P ratio in nucleic acid as 15.3:8.0 (see ref. 15), this loss of nucleic acid-P accounts for 0.24 g of N *i.e.* about half of the N lost when the extract is incubated. Evidently N compounds other than nucleic acid are also lost and loss by protease action is an obvious possibility. TRACEY[16] found more protease in wheat leaves than in the leaves of most other species of plants; I shall discuss in a later paper the autolysis that this brings about.

Fractionation of phosphorus compounds of the extract after precipitation

Table III shows the P content of various fractions of phosphorus compounds of the extract, when samples were precipitated by heat and TCA. There was more than twice as much P in a TCA precipitate as in a heat coagulum and almost all of this "extra P" was present as "extra NA-P". This "extra P" shows as an increase in "soluble P" after coagulation of the extract by heat, but after heating it was no longer in the form of nucleic acid precipitable by TCA.

TABLE III

FRACTIONATION OF PHOSPHORUS COMPOUNDS OF EXTRACTS PRECIPITATED BY HEAT AND TCA

Fractionation of phosphorus compounds of 2.0-ml samples of wheat leaf extract, diluted four fold, after precipitation by heat and TCA was done by the method described in the experimental section. The values given in the table have been calculated for undiluted extract.

Fractions	*Phosphorus in various fractions after precipitation*	
	by heat (g/l)	*by TCA (g/l)*
Soluble P	0.59	0.5
Total P in precipitate	0.076	0.165
Acid soluble P	0.004	—
Ethanol–ether soluble P	0.02	0.025
Nucleic acid-phosphorus (NA-P)	0.053	0.138

Enzyme inactivation by boiling

The enzymic digestion of nucleoprotein during heating was kept to a minimum by using the technique of heat coagulation described by PIRIE[14] to make stable nucleoprotein. The extract was added directly to boiling water in such a way that the boiling was continuously maintained during the addition and for 2 min afterwards. Total NA-P was measured in the coagulum produced in this way and in a TCA precipitate of the supernatant fluid after separating the coagulum. Table IV gives the results of one such experiment with values for a TCA precipitate and an ordinary heat coagulum, for comparison.

TABLE IV

TOTAL NUCLEIC ACID-PHOSPHORUS CONTENT OF A COAGULUM OF EXTRACT OBTAINED BY BOILING COMPARED WITH THAT OF A TCA PRECIPITATE AND AN ORDINARY HEAT COAGULUM

2.0-ml samples of wheat leaf extract, diluted four fold, were precipitated (a) by TCA, (b) by adding the extract direct to boiling water and (c) by heating the sample in a water-bath. Total NA-P was measured in the precipitates thus obtained and also in the TCA precipitates of the supernatant fluids got from the two types of heat coagulation. The values given in the table have been calculated for undiluted extract.

Methods of precipitation	*Total NA-P in*	
	precipitate (g/l)	*supernatant fluid (g/l)*
TCA precipitation	0.183	—
Coagulation by boiling	0.108	0.06
Coagulation by heating in a water-bath	0.063	0.006

The coagulum obtained by immediate boiling not only contained 70 % more NA-P than the ordinary heat coagulum, but the supernatant fluid from it also contained nucleic acid in a form precipitable by TCA, and in an amount comparable to that present in the ordinary heat coagulum. The total NA-P in the boiled coagulum and the supernatant fluid together equals about 90 % of that precipitable by TCA.

Precipitation by perchloric acid and Uranyl TCA

Perchloric acid and Uranyl TCA precipitated as much protein N as did TCA. The amount and nature of P precipitated by perchloric acid and TCA were almost similar, but the Uranyl TCA precipitate contained about 12 % more total P and about 25 % more NA-P than did a TCA precipitate. There was also 40 % less ethanol–ether soluble P in the Uranyl TCA precipitate than in a TCA precipitate.

DISCUSSION

The facts that a TCA precipitate contains more N than a heat coagulum and that more TCA precipitable N is lost when extracts are incubated, are indications that the two precipitates are of a different nature. As much of the "extra N" of TCA precipitates from the extract occurred in the supernatant fluid after removing the chloroplast fraction and as PIRIE[14] isolated a NP from a similar supernatant fluid of tobacco leaf extract, it seemed likely that more NP was precipitated by TCA than by heat. This expectation was confirmed by studying the nucleic acid content and more conclusively, by studying the phosphorus compounds of the two types of the precipitate.

This posed the question: Why is less NP precipitated by heat? TCA precipitation was done at 4° and the pH of the extract shifted from about 6 to that between 0.5 and 1.5, and under these conditions enzymic activity is presumably impossible. HOLDEN AND PIRIE[13] found no leaf RNase activity below pH 2.0 and PIRIE[17] showed that, at 0°, TCA precipitates of nucleoproteins do not show leaf RNase activity even in presence of citrate ions which are the most effective activators of this enzyme. But leaf RNase is not inactive when the leaf extract is coagulated by heating in a water-bath.

Nucleases are closely associated with leaf NP (see ref. 14) and leaf RNase survives heating at 53° for 3 min, complete inactivation occurring only at 88° (see ref. 13). Therefore the leaf RNase could hydrolyse considerable amounts of nucleic acid while the extract was being heated in the water-bath.

Pancreatic RNase[8] leaves a "resistant core" of nucleic acid precipitable by TCA, but leaf RNase renders all nucleic acid unprecipitable by TCA[14,18]. No nucleic acid precipitable by TCA was, therefore, released in a soluble form when the extract was coagulated in a boiling water-bath. However, when the extract was added directly to boiling water, the enzyme was quickly inactivated and some TCA precipitable nucleic acid was released in a soluble form because nucleoprotein was partly disrupted by heat[17,19].

TCA and perchloric acid behave similarly as precipitants of leaf proteins, but Uranyl TCA seems to precipitate some of the TCA soluble polynucleotides and to render some lipoprotein–polynucleotide complex insoluble in ethanol–ether. VENKATARAMAN AND LOWE[20] suggested that a lipoprotein–polynucleotide complex passes into ethanol phase because rat liver NP becomes partially soluble after precipitation by cold TCA. Probably some leaf NP may also dissolve in ethanol–ether after it is precipitated by cold TCA, but not after precipitation is done by uranyl TCA.

TCA-precipitated N consists not only of protein N, but also of nucleic acid N and the amount of N precipitated by TCA varies with the interval elapsing between making the leaf extract and doing the precipitation.

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N-(4-DIMETHYLAMINO-3,5-DINITROPHENYL)MALEIMIDE: A COLOURED SULFHYDRYL REAGENT*

ISOLATION AND INVESTIGATION OF CYSTEINE-CONTAINING PEPTIDES FROM HUMAN AND BOVINE SERUM ALBUMIN

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SUMMARY

The coloured sulfhydryl reagent, N-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM), has been synthesized and its reactions with cysteine and with the free SH groups of human and bovine serum albumin have been studied.

The DDPS-serum albumins, prepared from DDPM and serum albumins, were subjected to hydrolysis with pepsin. From the digests, S-DDPS-cysteine peptides were isolated by means of their yellow colour, and purified by adsorption on talc, paper ionophoresis and chromatography. The same amino acid sequence, Leu–Glu(NH_2)–Asp–Glu–Glu(NH_2)–Glu–CySH–Pro–Phe, has been found to occur in the peptides derived from bovine and from human serum albumin.

INTRODUCTION

The widespread use of FDNB[3] for labeling amino groups with the yellow DNP residue and for characterizing the N-terminal amino acids in polypeptide chains indicates the value of coloured reagents in protein chemistry. It was felt that it would be desirable to find a colour label which could be applied to protein sulfhydryl groups and which could be used for the identification of cysteine residues and for the investigation of amino acid sequences containing them.

In this work, N-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM) has been tested as a reagent for SH groups. More than ten years ago, the colourless

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Abbreviations: DDPM, N-(4-dimethylamino-3,5-dinitrophenyl) maleimide; DDPS, N-(4-dimethylamino-3,5-dinitrophenyl) succinimido-; NEM, N-ethylmaleimide; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; PCMB, *p*-chloromercuribenzoate; BSA, bovine serum albumin; HSA, human serum albumin. The abbreviations for the amino acid residues are those suggested by BRAND AND EDSALL[50].

* The use of DDPM as a label for SH groups in proteins was first reported at the Symposium on Sulphur in Proteins, Falmouth, Mass.,[1] May, 1958. When the work described in this paper was almost completed, BURLEY AND HAYLETT[2] published a paper on the preparation of DDPM and announced its use as a reagent for SH groups in wool.

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N-ethylmaleimide[4–6] was introduced into protein and peptide biochemistry and was shown to combine specifically with SH groups[7,8]. Subsequently, other maleimide derivatives such as N,N'-(1,3-phenylene)-bis-maleimide[9] and N-(4-hydroxy-1-naphthyl) maleimide[10] have also been used with success in protein chemistry and histochemistry, respectively, on account of their specific reaction with sulfhydryl groups. In contrast to the maleimide derivatives employed so far, DDPM is a coloured compound. It contains the yellow 4-dimethylamino-3,5-dinitrophenyl grouping which, a few years ago, became known in protein chemistry as part of the amino group reagents, 4-dimethylamino-3,5-dinitrophenyl isocyanate and isothiocyanate[11,12].

In order to explore the potentialities of DDPM, its reactions with the free SH groups of bovine and human serum albumin have been studied and the sequences of amino acids which surround the labeled cysteine residues have been determined.

MATERIAL AND METHODS

Commercial preparations of bovine serum albumin (Armour lots N 66706 and S 68108 and Sigma lot No. A 68–084) were used without further purification. Human serum albumin was kindly provided by the Academic Hospital, Groningen, in a 20 % solution. Pepsin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Subtilisin and carboxypeptidase were gifts of the late Professor K. LINDERSTRØM-LANG, Copenhagen, and of Dr. F. A. HOMMES, Groningen, respectively.

PCMB was obtained by synthesis[13] and purified by repeated precipitation from dilute sodium hydroxide solution by the addition of hydrochloric acid. NEM was purchased from Nutritional Biochemicals Corp.

For the preparation of S-DNP-cysteine from cysteine and FDNB, at pH 5.2, the method of ZAHN AND TRAUMANN[14] was used. On paper chromatograms developed with *n*-butanol/acetic acid/water (4:1:1, v/v/v) the compound had an R_F value slightly less than phenylalanine.

DDPM was prepared from 4-dimethylamino-3:5-dinitroaniline which was readily obtained by following the procedure of EVANS AND REITH[11]. The last step of the synthesis, however, was carried out in a different way: N-acetyl-4-dimethylamino-3,5-dinitroaniline (10 g) was heated for 2 h on a boiling water-bath with a mixture of conc. HCl, water and 96 % ethanol (75 ml each). After cooling the mixture was diluted with water (150 ml), the precipitate filtered off, washed with water and dried (6.3 g), m.p. 157–158°. On recrystallisation from dil. ethanol the red needles of 4-dimethylamino-3:5-dinitroaniline had a m.p. 157.5–158°. (AYLING *et al.*[15] reported 157°, REITH AND WALDRON[12] 155–156°, BURLEY AND HAYLETT[2] 160°.) (Found: C, 42.7; H, 4.65; N, 24.7. $C_8H_{10}N_4O_4$ requires C, 42.5; H, 4.5; N, 24.8 %.)

N-(4-Dimethylamino-3:5-dinitrophenyl) maleamic acid

The above amine (10 g) was dissolved in anhydrous ether (65 ml) and maleic anhydride (4.85 g) added. The clear solution was left overnight in the dark, with exclusion of moisture. The orange-red crystals which had then separated, were filtered and dried (14.2 g). Recrystallized from ethanol–water, the needles had a m.p. 178–179°. (BURLEY AND HAYLETT[2] reported 176°.) (Found: N, 17.3; $C_{12}H_{12}N_4O_7$ requires N, 17.3 %.)

DDPM (formula I)

This was obtained by heating the maleamic acid derivative (10 g) with acetic anhydride (75 ml) and anhydrous sodium acetate (1.5 g) for 15 min on a boiling water-bath, with exclusion of moisture. The solution was then concentrated *in vacuo* at 35° to a volume of about 25 ml and diluted with water (250 ml). After standing for a few hours at room temperature, the crude product was filtered, washed with water, and dried (7.9 g). Twice recrystallized from acetone–water, the long thin orange needles had a m.p. 179–181°. (BURLEY AND HAYLETT[2] reported 181°.) (Found: C 47.4; H, 3.4; N, 18.1. $C_{12}H_{10}N_4O_6$ requires C, 47.1; H, 3.3; N, 18.3 %.) DDPM is sparingly soluble in water, but dissolves in acetone, ethanol, dimethylformamide and acetic acid, and aqueous solutions can be obtained by dilution with water. At alkaline pH values DDPM (like NEM[46]) readily hydrolyses to the corresponding maleamic acid.

Reaction of DDPM with cysteine

The solutions of 610 mg DDPM in 20 ml acetone and of 351 mg cysteine hydrochloride in 20 ml deoxygenated water were mixed. The mixture was adjusted to pH 5 by the addition of solid $NaHCO_3$, left at room temp. for 90 min, and evaporated to dryness *in vacuo* at a temperature not exceeding 35°. The solid material was washed with a little water and dried (0.81 g, 95 %). When triturated with warm acetone, most of the yellow substance remained undissolved. This compound, S-DDPS-cysteine (formula II), is slightly soluble in cold water. It readily dissolves in glacial acetic acid and in dimethylformamide. It may be precipitated from its solution in acetic acid with ether. For purification, some of the material was suspended in aqueous ethanol, dissolved by careful dropwise addition of 1 *N* HCl in the cold, and reprecipitated by slowly adding sodium acetate. The orange, amorphous substance which separated was washed with a little water and dried *in vacuo* over P_2O_5, at 78°. M.P. 169–170°. (Found: C, 40.71; H, 4.50. $C_{15}H_{17}O_8N_5S \cdot H_2O$ requires C, 40.45; H, 4.30 %.) When subjected to paper electrophoresis in 2 *N* acetic acid–0.6 *N* formic acid at pH 2 (ref. 16), it moved towards the cathode as an intense yellow band which turned purple on treatment with ninhydrin. Ionophoresis in pyridine–acetic acid buffer at pH 4.7 (ref. 24) indicated that, at this pH, the compound had no net charge. On paper chromatograms developed with the upper phase of a mixture of *n*-butanol, acetic acid and water (4:1:5, v/v/v) it showed an R_F value of 0.68.

Cleavage by silver ions of the thioether bond in S-DDPS-cysteine

A solution of 3 mg S-DDPS-cysteine in 1.25 ml 60 % acetic acid was added to a solution of 20 mg silver sulphate in 2.5 ml warm water. The mixture was heated at 50° for 9 h and subsequently concentrated *in vacuo*. The dry residue was treated for 20 min at room temp. with a mixture of 0.75 ml 87 % formic acid and 0.075 ml 30 % hydrogen peroxide. The performic acid was then removed *in vacuo* at a temperature of 30° and the residual material twice taken up with water and brought to dryness again to remove traces of hydrogen peroxide; 10 ml of warm water (60°) and 1 drop 20 % sulphuric acid were added, and hydrogen sulphide was bubbled through the solution. The precipitated silver sulphide was centrifuged off and washed with water in the centrifuge. The clear supernatants were combined and freed of sulphuric acid by extraction with a chloroform solution of tri-*n*-octylamine[17]. As shown by paper chromatography, the aqueous solution was rich in cysteic acid.

Rearrangement of S-DDPS-cysteine on exposure to alkali

Even a short exposure to mild alkali (dilute sodium carbonate) has been found to transform S-DDPS-cysteine into another yellow compound which is sparingly soluble in dilute acid and does not give a colour reaction with ninhydrin. On paper ionophoresis at pH 2 it does not migrate, while at pH 4.7 it moves towards the anode with a mobility about one half that of glutamic acid. R_F (in *n*-butanol–acetic acid–water, 4:1:5) 0.86. Recrystallised from acetone–petrol ether it melted at 245–247° (decomp.) (Found: C, 41.65; H, 4.05; N, 15.82. $C_{15}H_{17}O_8N_5S$ requires C 42.15; H, 4.01; N, 16.39 %.) This rearrangement product has been assigned the structure of a thiazane derivative (formula III).

Cleavage by mercuric ions of the thioether bond in the thiazane derivative III

Treatment of III with silver ions in acetic acid at 50° followed by performic acid oxidation did not yield cysteic acid. The latter could be obtained, however, by heating III with mercuric chloride in dilute hydrochloric acid, prior to oxidation. A solution of 2.25 mg III and 1.2 mg $HgCl_2$ in 0.25 ml 0.025 *N* HCl was heated in a sealed tube at 105° for 21 h. The reaction mixture was diluted with 0.25 ml water and extracted three times with 0.5 ml ether. The orange-coloured ether layer was discarded and the nearly colourless aqueous layers oxidised with performic acid. Paper chromatography showed cysteic acid to be the ninhydrin-positive material present.

Acid hydrolysis of S-DDPS-cysteine and of the thiazane derivative III

When S-DDPS-cysteine and the compound III were subjected to acid hydrolysis in sealed tubes (5.7 *N* HCl, 15 h, 105°) and the hydrolysates were examined by paper chromatography (*n*-butanol–acetic acid–water, 4:1:5), the same pattern of spots was obtained. In addition to a set of yellow and orange spots which did not give a colour reaction with ninhydrin and had R_F values in the range between 0.80 and 0.95, 3 ninhydrin-positive spots were found, one of them (R_F 0.16) being by far the strongest; this was indistinguishable from that given by synthetic 2-amino-2-carboxyethyl-mercaptosuccinic acid[16,9]. The two weaker spots had lower R_F values and occurred in variable amounts. The slowest one (R_F 0.07) was identified as cysteic acid. The same set of three ninhydrin-positive spots was also obtained when 2-amino-2-carboxyethyl-mercaptosuccinic acid was subjected to acid hydrolysis. In paper chromatography, using the butanol–acetic acid–water system, the 2-amino-2-carboxyethyl-mercaptosuccinic acid spot (R_F 0.16) can easily be mistaken for aspartic acid (R_F 0.14). With *n*-butanol–pyridine–water (1:1:1), however, the R_F values are markedly different (0.09 and 0.19, resp.). On paper ionophoresis, at pH 4.8, 2-amino-2-carboxyethyl-mercaptosuccinic acid migrates towards the anode more rapidly than aspartic acid.

Titration of SH groups in serum albumins

The SH groups were titrated spectrophotometrically, according to BOYER[18], with PCMB in 0.1 *M* phosphate pH 6.9 or in 0.33 *M* acetate buffer pH 4.6. The PCMB solution was added with an Agla micrometer syringe, in 20-μl portions of a $5 \cdot 10^{-4}$ *M* solution, and the extinction at 250 mμ measured after each addition. Alternatively, titrations were carried out with 10^{-3} *M* NEM in 0.1 *M* phosphate buffer of pH 6.7 (ref. 19–20), readings being taken at 300 mμ. The concentrations of bovine and human serum albumins in their solutions were also determined spectrophotometrically at 280 mμ, using the extinction coefficients, $[E]_{1\,\text{cm}}^{1\%}$, of 6.6 and of 5.3, respectively[21].

Reaction of bovine serum albumin with DDPM

To solutions of BSA (10^{-5} *M*) in 0.33 *M* acetate buffer, pH 4.6, graded volumes of a 0.01 % solution of DDPM in a mixture of equal parts of 96 % ethanol and acetate buffer were added. The mixtures were left at room temp. for 1 h. Subsequently, unreacted SH groups were determined by titration with PCMB. After 1 h's contact of BSA with a 1.5-fold molar excess of DDPM, free SH groups were no longer detectable.

Preparation of DDPS-bovine serum albumin

15 mg DDPM were dissolved in 50 ml of ethanol. An equal volume of 0.33 *M* acetate buffer, pH 4.6, was added, and the mixture was slowly run into 100 ml of a 1 % solution of BSA in acetate buffer. After standing overnight at room temp., the clear solution was concentrated *in vacuo* at 35°. DDPS-bovine serum albumin was precipitated by the addition of 10 volumes of ethanol and, after a few hours, collected by centrifugation, washed with ethanol and acetone to remove excess DDPM, and dried.

Enzymic hydrolysis of DDPS-bovine serum albumin and isolation of DDPS-cysteine peptides

DDPS–BSA (1 g) was dissolved in 95 ml 0.01 *N* HCl. A solution of 10 mg pepsin in 5 ml 0.01 *N* HCl was added, the mixture adjusted to pH 2.1 and incubated at 37° for 24 h.

A preliminary fractionation of the digest was effected by passing it through a talc column. Talc (24 g) was suspended in a mixture of 2 *N* HCl and ethanol (1:1, v/v, heated on a steam-bath for 2 h and repeatedly washed with water by decantation until the washwater was no longer acidic. The talc was re-suspended in water and poured into a column 10 cm high and 1.5 cm in diameter. The digestion mixture was filtered through the talc, with gentle suction, followed by water. The yellow-coloured peptides, which had been adsorbed on the upper parts of the column, were subsequently eluted from the talc by passing through it 50 % aqueous ethanol containing 2 % acetic acid. The yellow eluate was reduced to dryness in a desiccator.

The yellow material obtained (45 mg) was further purified by subjecting aliquot parts to high-voltage (1500–2000 V) ionophoresis on paper (Schleicher and Schüll No. 2043a, 17 × 60 cm, or Whatman 3MM, 13 × 39 cm), using a formic acid–acetic acid buffer[16] of pH 2 (Fig. 1). The yields of the yellow-coloured peptides *A*, *B*, and *C* varied to some extent in different experiments. *B* usually exceeded *A*, and *C* was only a faintly coloured, minor component. When the paper strips were sprayed with ninhydrin and heated, *B* turned brown–violet while *A* did not react. Ninhydrin also revealed a number of contaminating peptide bands (not indicated in Fig. 1) which had no yellow colour and were mainly located in the region of *C*.

Peptides *A* and *B* were eluted from the paper strips with 10 % aqueous acetic acid. The eluates were taken to dryness. In most experiments the peptides were further subjected to descending paper chromatography. When B was run in the solvent mixture, tertiary amyl alcohol–pyridine–water (7:7:6, v/v/v): for 15 h, after an equilibration period of 24 h, the main yellow-coloured and ninhydrin-reactive material had an R_F value of approx. 0.50. It was neatly separated from other faintly yellow-coloured or weakly ninhydrin-reacting components.

Structural investigation of the DDPS-cysteine peptide B *obtained from BSA*

Total hydrolysis: This was performed on peptide *B* with 5.7 *N* HCl in a sealed capillary tube for 16 h at 105° and yielded the following amino acids, identified by paper chromatography: Leu (xx), Phe (xx), Pro (xx), Glu (xxxxx), Asp (xx) and "Cys" (xx). "Cys" indicates the mixture of degradation products, arising from DDPS-cysteine on hydrolysis with HCl, which gives on paper chromatograms sprayed with ninhydrin a characteristic pattern of yellow and purple spots. The presence of aspartic acid was confirmed by paper ionophoresis at pH 4.7, and the occurrence of leucine rather than isoleucine by paper chromatography in pyridine–tertiary amyl alcohol–water (7:7:6, v/v/v)[47].

N-terminal residue: This was determined using the DNP-method. The peptide was reacted with FDNB in the presence of trimethylamine[22]. The DNP-derivative obtained was hydrolysed with a mixture of glacial acetic acid and conc. HCl (1:1) for 70 min at 105°. The ether-soluble yellow compounds present in the hydrolysate were characterised by paper chromatography using the phenol–isoamyl alcohol–ammonia solvent described by BISERTE AND OSTEUX[23]. The main yellow spot had an R_F value of 0.32 and was identified as DNP-Leu. A minor yellow component with R_F 0.22 was found to yield DNP-Leu and Glu when hydrolysed with 5.7 *N* HCl (16 h, 105°).

The C-terminal residue: This was identified with the help of carboxypeptidase. A solution (0.55 ml) containing 3 μg enzyme and 500 μg peptide *B* was brought to pH 8.0 by the addition of trimethylamine and incubated at 37°. At various times 0.05-ml samples of the incubation mixture were withdrawn and investigated by paper chromatography. Phenylalanine was found to be slowly released. Even after 24 h at 37° not even a trace of an amino acid other than phenylalanine could be detected.

Cleavage by subtilisin: A solution (0.7 ml) containing 5.6 mg *B* and 0.28 mg subtilisin was adjusted to pH 7.7 with $NaHCO_3$ and incubated at 37° for 20 h. Two drops of glacial acetic acid were added to stop the enzymic reaction and the digestion mixture was evaporated to dryness *in vacuo*. Samples of the digested peptide mixture were subjected to high-voltage paper ionophoresis (Figs. 2(a) and (b)). Four peptides which separated from each other were eluted from the paper and investigated as summarized in Table I.

On paper chromatograms of hydrolysates of the peptides Bb_1 and Bb_2 the glutamic acid spots were strikingly stronger than the spots of the other amino acids, indicating that more than one glutamic acid residue was present (Table I). This was confirmed by semi-quantitative assay. Hydrolysates of the peptides Bb_1 and Bb_2 were allowed to react with FDNB in aqueous alcohol containing $NaHCO_3$. The reaction mixtures were diluted with water and extracted with ether to remove excess FDNB. The aqueous solutions were acidified and extracted with ether again. The ether extracts which contained the DNP-amino acids were taken to dryness and dini-

TABLE I

PEPTIDES OBTAINED BY DIGESTION OF THE S-DDPS-CYSTEINE PEPTIDE *B* WITH SUBTILISIN

Peptide	*Colour*	*Colour reaction with ninhydrin*	*Amino acids obtained on acid hydrolysis*	*Dinitrophenylation followed by acid hydrolysis*	
				*DNP-amino acids**	*Free amino acids*
Ba	none	purple	Leu(xxx), Glu(xxx)	DNP-Leu	Glu
Bb_1	yellow	brownish-purple	Phe(xx), Pro(xx), Glu(xxx), "Cys"(x)	DNP-Glu	Phe(xx), Pro(xx), Glu(xx), $CySO_3H$(xx)
Bb_2	yellow	none	Phe(xx), Pro(xx), Glu(xxx), "Cys"(x)	none	Phe(xx), Pro(x), Glu(xxxx), $CySO_3H$(xx)
Bc	none	purple	Glu(xxx), Asp(xx)	DNP-Asp	Glu

* The DNP-amino acids were characterized by paper chromatography in the solvents amyl alcohol–phenol–ammonia[23] or tert. amyl alcohol–phthalate buffer pH 6 (ref. 25) and by cleavage with ammonia[26], the resulting free amino acids likewise being identified by chromatography.

trophenol was removed from the residue by sublimation *in vacuo*[27] at 78°. The mixtures of DNP-amino acids were subjected to descending chromatography on Schleicher and Schüll paper No. 2045 bM. The solvent was *n*-butanol saturated with water. Reference samples containing various known amounts of DNP-glutamic acid, DNP-proline and DNP-phenylalanine were run side by side with the unknown mixtures. The yellow spots corresponding to the DNP-derivatives of Glu, Pro, and Phe were cut out, eluted with 5 ml of 1 % $NaHCO_3$ for 15 min[28] at 55–60° and the extinctions of the eluates determined at 360 mμ (for DNP–Glu and DNP–Phe) and 385 mμ (for DNP–Pro) in a Beckman spectrophotometer. From the readings the molar ratios of Phe:Pro:Glu were calculated to be 1.12:1.12:2.00 for peptide Bb_1 and 1.07:0.93:2.00 for Bb_2. Partial acid hydrolysis (conc. HCl, 72 h, 37°) of the peptides Bb_1 and Bb_2 gave complex mixtures which were fractionated by paper ionophoresis at pH 6.5 (Fig. 5) followed by descending paper chromatography using the solvent system *n*-butanol–acetic acid–pyridine–water (4:1:1:5, v/v/v/v)[51]. The results are summarized in Table II.

Investigation of the DDPS-cysteine peptide A obtained from BSA

Peptide *A* (Fig. 1) does not give a colour reaction with ninhydrin nor has it been possible to identify a free N-terminal amino acid residue using the DNP technique. *A* appears to contain the same amino acids as *B*. Cleavage with subtilisin gave three split products closely resembling or identical with the peptides *a*, b_2 and *c* as derived from *B*.

Investigation of DDPS-cysteine peptides obtained from HSA

Human serum albumin was allowed to react with DDPM in the same way as described above for BSA except for the use of a 0.1 *M* phosphate buffer of pH 6.7 instead of an acetate buffer of pH 4.6. Peptic cleavage of the DDPS derivative of HSA, followed by the purification procedure indicated above, gave two DDPS-cysteine peptides (*B′* and *A′*) which were identical with *B* and *A*, respectively, as regards their electrophoretic mobilities, their reactions with ninhydrin, their R_F values, their amino acid compositions, their N- and C-terminal residues, and the products of their cleavage by subtilisin.

TABLE II

AMINO ACIDS AND PEPTIDES OBTAINED FROM THE S-DDPS-CYSTEINE PEPTIDES Bb_1 AND Bb_2 BY PARTIAL ACID HYDROLYSIS AND SEPARATED BY PAPER IONOPHORESIS AND PAPER CHROMATOGRAPHY

Ionophoresis fractions (Fig. 5)	*Colour*	*Colour reaction with ninhydrin*	*Intensity*	*Spots obtained on paper chromatography*	R_{Phe}**	*Colour reaction with ninhydrin*	*Intensity*	*Amino acids obtained on total acid hydrolysis, identified by paper chromatography*	*Inferred sequence*
Bb_1*	brown	brown–purple	xxxx	a	0.08	purple	xx	"Cys"	
				b	0.19	purple	xxx	"Cys"	
				c	0.31	purple	xx	Pro, "Cys"	Cys–Pro
				d	0.42	yellow	xx	Pro	
				e	0.76	violet	trace		
				f	1.00	violet	xx	Phe	
				g	1.21	yellow	xxx	Phe, Pro	Pro–Phe
Bb_2	—	purple	xxx	a	0.08	violet	xx	Pro, Glu, "Cys"	Glu–Cys–Pro
				b	0.13	violet	xxx	Pro, Glu(!), "Cys"	Glu–Glu–Cys–Pro
				c	0.18	purple	xxxx	Glu	
				d	0.22	violet	xxx	Phe, Pro, Glu, "Cys"	Glu–Cys–Pro–Phe
				e	0.37	pink	x	Phe, Pro, "Cys"	Cys–Pro–Phe
Bb_3	—	purple	xx	a	0.05	violet	xxx	Glu(!), "Cys"	Glu–Glu–Cys
				b	0.08	purple	xx	Glu, "Cys"	Glu–Cys
				c	0.13	purple	xx	Glu	Glu–Glu
				d	0.20	purple	trace		
									Glu–Glu–Cys–Pro–Phe

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* This fraction showed, on paper chromatography, coloured spots, which were derived from the DDPS moieties of Bb_1 and Bb_2.

** R_{Phe} values of reference substances in the system *n*-butanol–pyridine–acetic acid–water (4:1:1:5, v/v/v/v): 2-amino-2-carboxyethyl–mercaptosuccinic acid, 0.08; Glu–Glu, 0.13; Glu, 0.19; Pro, 0.43; Phe, 1.00.

Preparation of DNP-bovine serum albumin

A solution of 1 g BSA in 15 ml water was adjusted to pH 9.3 with dil. NaOH and a solution of 3.5 mg chlorodinitrobenzene in 5 ml 96 % ethanol was added. The clear mixture was left at 37° for 1.5 h and then poured into 200 ml 96 % ethanol. A yellow precipitate formed when conc. HCl was added to bring the pH to 3.5. After standing for 2 h, it was collected by centrifugation, washed and dried.

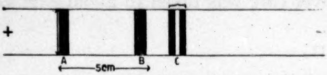

Fig. 1

Fig. 1. Paper ionophoresis of DDPS-cysteine peptides, obtained by pepsin digestion of DDPS-bovine serum albumin. In formic acid–acetic acid, pH = 2.0 (ref. 16), 2 h, 50 V/cm, 22 mA.

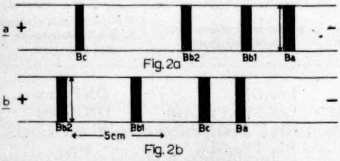

Fig. 2a

Fig. 2b

Fig. 2. Paper ionophoresis of the mixture of split products, obtained by digesting the S-DDPS-cysteine peptide *B* with subtilisin. (a) In pyridine–acetic acid, pH = 4.7 (ref. 24), 2.5 h, 25 V/cm, 36 mA. (b) In formic acid–acetic acid, pH = 2.0 (ref. 16).

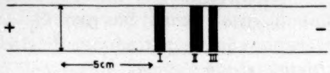

Fig. 3

Fig. 3. Paper ionophoresis of DNP-peptides, obtained by pepsin digestion of DNP-bovine serum albumin. In formic acid–acetic acid, pH = 2.0 (ref. 16), 2 h, 40 V/cm, 24 mA.

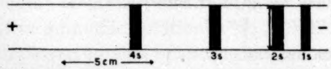

Fig. 4

Fig. 4. Paper ionophoresis of the peptide mixture, obtained by digesting the S-DNP-cysteine peptide *I* with subtilisin. In pyridine–acetic acid, pH = 4.6 (ref. 24), 2 h, 25 V/cm, 27 mA.

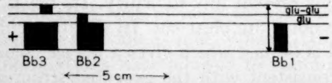

Fig. 5

Fig. 5. Paper ionophoresis of the mixture of split products, obtained by partial hydrolysis of the S-DDPS-cysteine peptides Bb_1 and Bb_2. In pyridine–acetic acid, pH = 6.5 (ref. 24), 1.5 h, 40 V/cm, 27 mA. For comparison glutamic acid and the dipeptide glutamyl-glutamic acid were simultaneously subjected to paper ionophoresis.

An S-DNP-cysteine peptide from DNP-bovine serum albumin

The dinitrophenylated BSA was digested with pepsin and the digest filtered through a talc column (15 cm high and 1.8 cm in diameter) under slightly reduced pressure. Material having a faintly yellow colour was adsorbed on the upper three-quarters of the talc. The column was washed with 7.5 ml water and then eluted with 2 % ammonia in 50 % aqueous ethanol. The yellow peptides slowly moved down the column, forming a sharp band. The lemon-coloured eluate was lyophilised and subjected to ionophoresis on paper (Fig. 3). Besides a considerable number of colourless peptides which only showed up after treatment with ninhydrin and are not indicated in the figure, three yellow bands were obtained. All of them gave a colour reaction with ninhydrin (I, grey–blue, weak; II, blue–green, strong; III, grey–purple, very weak). On acid hydrolysis, only I gave S-DNP-cysteine. In the other two peptides, II and III, the DNP residues were bound to groups other than SH groups. The S-DNP-cysteine peptide I was further purified by chromatography in the same manner as described above for DDPS-cysteine peptides. It had a R_F value of approx. 0.50 and was clearly separated from contaminating ninhydrin-positive substances, all of which had lower R_F values. It could most readily be located inspection of the chromatograms in u.v. light when it showed up as a dark spot.

On total hydrolysis with acid, peptide I gave the following amino acids: Leu (xx), Phe (xx), Pro (xx), Glu (xxxx), Asp (xx), S-DNP-cysteine (x), and cysteic acid (x). Proteolytic cleavage of the peptide with subtilisin was slow and incomplete even after prolonged incubation. The digestion mixture was subjected to paper ionophoresis (Fig. 4). The separated peptides were eluted and further examined as recorded in Table III.

In all operations involving DNP derivatives care was taken to avoid exposure to bright light.

TABLE III

PEPTIDES OBTAINED BY HYDROLYSIS OF THE S-DNP-CYSTEINE PEPTIDE *I* WITH SUBTILISIN

Peptide	*Colour*	*In u.v. light*	*Colour reaction with ninhydrin*	*Amino acids obtained on acid hydrolysis*	*DNP-amino acid obtained by dinitrophenylation and hydrolysis*
1s	none	—	purple	Leu,Glu	DNP-Leu
*2s**	faint yellow	dark	grey-purple	Leu,Phe,Pro,Asp, Glu**,S-DNP-Cys	DNP-Leu
3s	faint yellow	dark	none	Phe,Pro,Glu, S-DNP-Cys	none
4s	none	—	purple	Asp,Glu	DNP-Asp

* Peptide *2s* is unchanged peptide *I*.
** The glutamic acid spot was strikingly stronger than all the other amino acid spots.

RESULTS AND DISCUSSION

The preparation of DDPM (formula I) does not present any difficulty. Its reaction with cysteine, however, has been found to be not quite clear-cut. In slightly acidic solutions a yellow-coloured neutral compound is formed which gives a purple colour reaction with ninhydrin and has properties consistent with the expected structure of a S-[N-(4-dimethylamino-3:5-dinitrophenyl)-succinimido]-cysteine (formula II). At an alkaline pH, however, and even in neutral solutions II readily rearranges into an acidic compound which does not react with ninhydrin and to which is assigned formula III. The rearrangement is presumed to be initiated by the removal of a proton from the NH_3^+ group of DDPS-cysteine (II) and to proceed via an intramolecular acylation of the NH_2, the succinimide ring being opened and a thiazane ring formed*.

DDPS-cysteine and the thiazane derivative arising from it in alkaline solution were subjected to the action of silver sulphate in acetic acid followed by oxidation with performic acid. This procedure had previously been used to break the thioether bonds in cytochrome *c*[29] and in hemopeptides derived from it[30]. With DDPS-cysteine this treatment was found to give a good yield of cysteic acid, whereas with its rearrangement product none was produced. Cysteic acid could be obtained from the thiazane derivative, however, by reacting it with mercuric ions, under conditions (0.025 *N* HCl, 105°, 21 h) where cleavage of the thioether linkage was accompanied by hydrolytic opening of the CO–NH bond, followed by performic acid oxidation.

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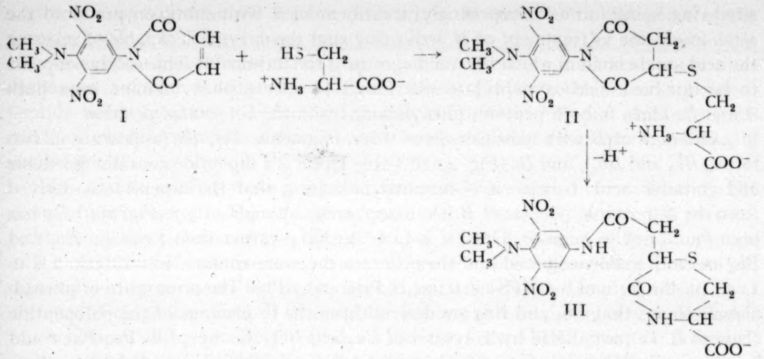
* During the preparation of this manuscript, Professor J. S. Fruton kindly informed us that experiments in his laboratory (D. G. Smyth, A. Nagamatsu and J. S. Fruton, unpublished) have shown that the cysteine adduct of N-ethyl maleimide, upon exposure to alkali, is transformed into a thiazane derivative corresponding to III.



These findings are in good agreement with the formulae II and III, respectively, assigned to the two compounds.

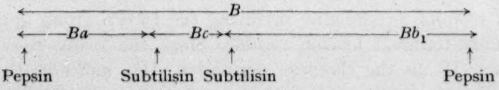
Bovine and human serum albumin were used to examine the reaction of DDPM with SH groups of cysteine residues in proteins. It is well known that in the absence of denaturing agents approximately two-thirds of one SH group per molecule of bovine or human serum albumin will react with SH reagents such as silver in Tris or in ammonia solution[31, 31A], PCMB[18], or NEM[19]. With the serum albumin samples employed in this investigation, titrations with PCMB gave values of 0.72 SH/mole BSA (at pH 4.6), 0.65 SH/mole BSA (at pH 6.9), and 0.60 SH/mole HSA (at pH 6.9). When HSA was allowed to react with NEM, 0.63 SH/mole were found to have combined within 45 min. When DDPM was added to solutions of HSA or BSA and the mixtures were allowed to stand for 1 h at room temperature, 1.5 moles of the maleimide reagent per one mole of albumin were found adequate for a quantitative reaction; subsequent titration of the serum albumins with PCMB gave evidence for the complete absence of residual easily accessible SH groups. The necessity for using a SH reagent in excess had been reported before for NEM[20].

When the S-DDPS derivative of BSA was subjected to hydrolysis with pepsin and the digestion mixture worked up, two S-DDPS-cysteine peptides, *A* and *B*, were mainly obtained (Fig. 1). The relative amounts of *A* and *B* were different in different experiments, and there are indications that *A* may arise from *B* in the course of the isolation procedures. Both peptides had the same amino acid composition (Leu, Phe, Pro, Glu, Asp, Cys) and, on enzymic hydrolysis, gave identical split products, which indicated that they had the same sequence of amino acids. *B* gave a purple colour with ninhydrin and readily reacted with FDNB. Acid hydrolysis of DNP-*B* gave DNP-Leu-Glu. *A*, on the other hand, did not react with ninhydrin nor could a DNP derivative be obtained from it. It obviously lacks a free α-NH_2 group. An unexpected reaction presumably involving the DDPS group appears to have masked the amino-terminal leucine residue. Since the ready re-arrangement of S-DDPS-cysteine (II) to the thiazane derivative (III) indicates that the DDPS group has acylating properties, it may be presumed that the DDPS moiety in the peptide *B* is able to acylate the free N-terminal α-amino group. It appears likely that this acylation occurs intramolecularly rather than intermolecularly, a largesized ring being formed. Surprisingly, treatment of *A* with subtilisin produced the same fragments as treatment of *B*, indicating that the enzyme is capable of severing the acid amide bond in which the amino group of the terminal leucine residue appears to be involved. Carboxypeptidase was found to release phenylalanine from both *A* and *B*. Thus, in both peptides phenylalanine is in the C-terminal position.

Cleavage of *B* with subtilisin gave three fragments, *Ba*, *Bb* (appearing in two forms, Bb_1 and Bb_2), and *Bc* (Fig. 2 and Table I). *Ba* is a dipeptide containing leucine and glutamic acid. Leucine is N-terminal indicating that the dipeptide is derived from the N-terminal portion of *B*. On ionophoresis at a pH of 4.7 (Fig. 2(a)) *Ba* has been found not to migrate. Thus, it is Leu-Glu(NH_2) rather than Leu-Glu. Bb_1 and Bb_2 are both yellow-coloured and they contain the same amino acid residues: 2 Glu, 1 Cys (in the form of S-DDPS-cysteine), 1 Pro, and 1 Phe. The occurrence of phenylalanine shows that Bb_1 and Bb_2 are derived from the C-terminus of the polypeptide chain of *B*. In partial acid hydrolysates of Bb_1 and Bb_2, the dipeptide Pro-Phe could be identified. Thus, the C-terminal phenylalanine residue is preceded by proline. (This finding is consistent with the slow release of phenylalanine from *B* under the action of carboxypeptidase. Proline residues adjacent to C-terminal phenylalanine have previously been reported to retard the release of the latter by carboxypeptidase while C-terminal proline residues themselves are known to be entirely resistant to the action of this enzyme[32].) The entire pentapeptide sequence in the peptides Bb_1 and Bb_2 can be easily deduced from the results of partial acid hydrolysis summarised in Table II. Bb_1 and Bb_2 show the following differences: Bb_1 gives a purple colour reaction with ninhydrin while Bb_2 does not react; using the DNP technique, one of the two glutamic acid residues of Bb_1 has been proved to be N-terminal, while Bb_2 does not react with FDNB; furthermore, paper ionophoresis reveals that Bb_2 is more acidic than Bb_1. These findings can be accounted for by assuming that the N-terminal residue in Bb_1 is glutamine rather than glutamic acid, and in Bb_2 is pyroglutamic (pyrrolidone carboxylic) acid. Glutamine residues which occupy a N-terminal position in peptides are known to undergo readily a cyclisation to pyroglutamic residues[33, 34]. In this transformation the free α-amino group is lost which is responsible for the reactions with ninhydrin and FDNB and which, at the pH used in ionophoresis, bears a positive charge. *Bc* is an acidic dipeptide composed of aspartic and glutamic acid residues. It must be derived from the middle part of the polypeptide chains of *A* and *B*. Aspartic acid is N-terminal. Carboxypeptidase has been found to split *Bc* into free aspartic acid and glutamic acid. Therefore, the free acids rather than their amides, glutamine and asparagine, appear to build up the dipeptide. The cleavage of dipeptides, using relatively high concentrations of carboxypeptidase, has been reported previously[52, 53].

From the results obtained the following amino acid sequence can be deduced:

. . .–Leu–Glu(NH_2)–Asp–Glu–Glu(NH_2)–Glu*–Cys*–Pro–Phe– . . .



Cys* signifies the cysteine residue which has been labeled by reaction with DDPM. It has not been established whether the amino acid residue symbolized by Glu* is a glutamyl or a glutaminyl residue. The arrows below the formula point to the peptide bonds which have been found susceptible to cleavage by the proteolytic enzymes, pepsin and subtilisin. The split linkages conform to the known specificities of the enzymes used: subtilisin has previously been reported to attack, among others, peptide bonds involving the carboxyl groups of glutamine[35, 36] and glutamic acid[37] residues, whereas pepsin is known to split bonds adjacent to aromatic amino acid residues[38-40].

In order to confirm these results, experiments were carried out to label the SH group of bovine serum albumin with a reagent other than DDPM. For this purpose, 1-chloro-2:4-dinitrobenzene was chosen. It was allowed to react with the albumin in a molar ratio of approximately 1.2:1. When the dinitrophenylated protein was digested with pepsin and the digest worked up, a S-DNP-cysteine peptide, *I*, was obtained (Fig. 3). The yield of this peptide was low and its colour very faint, and it was accompanied by two DNP-peptides which carried the DNP group on functional groups other than SH. This shows that the specificity of chlorodinitrobenzene for SH groups, even under relatively favourable conditions, is inferior to that of DDPM. (The same is true for the use of FDNB as a SH reagent. ZUBER, TRAUMANN AND ZAHN[48] who studied the reaction of FDNB with wool, at a pH of 5.2, were unable to effect specific and complete dinitrophenylation. Treatment of serum albumin with FDNB at pH 5.5 has been reported by ŠORM, KÖRBL AND MATOUSEK[49] to yield a yellow product which contained three dinitrophenyl residues per mole of albumin as estimated spectrophotometrically; this compares well with our finding of three DNP peptides in the peptic digest of serum albumin treated with chlorodinitrobenzene.) The structural investigation of the S-DNP-peptide *I* has given results analogous to those obtained with the DDPS-cysteine peptide *B* and has thus been valuable in corroborating the conclusions drawn above (Table III, Fig. 4). Attention may be drawn to the fact that in the subtilisin digest of peptide *I* a S-DNP-cysteine peptide, 3*s*, quite analogous to the S-DDPS-cysteine peptide, Bb_2, was found. 3*s* was negatively charged at a pH of 4.7, gave no colour reaction with ninhydrin and appeared not to have a free α-amino group. It may be assumed that the lack of an α-amino group in both 3*s* and Bb_2 is similarly due to the cyclisation of a N-terminal glutamine residue resulting in a pyroglutamic acid residue.

When the S-DDPS derivative of human serum albumin was degraded with pepsin in the same manner as the derivative of bovine serum albumin, exactly the same DDPS-cysteine peptides were obtained. The finding that the amino-acid sequences in the S-DDPS-cysteine peptides *B* (from BSA) and B′ (from HSA) were identical was not expected. The two serum albumins are known to be similar but they have been reported to differ with respect to amino acid composition[41, 42], N-terminal[43] and C-terminal amino acid sequences[44], u.v. light absorption[21], and the composition of peptides in partial hydrolysates[45].

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DIE ERSCHÖPFENDE REINIGUNG VON AKTIN-PRÄPARATEN ZAHL UND ART DER PHOSPHATHALTIGEN PROSTHETISCHEN GRUPPEN VON G- UND F-AKTIN

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(Eingegangen am 2. April 1960)

SUMMARY

Exhaustive purification of actin preparations. Number and kind of phosphate containing prosthetic groups of G- and F-actin

1. Previous investigations on the nucleoside phosphate content of G- and F-actin have all been carried out with unpurified or little purified protein preparations. It has never been tested whether the purification was complete or whether the purification itself inactivated the preparation.

2. In F-actin solutions prepared according to STRAUB the content of adenine decreases by repeated ultracentrifugal sedimentation or Mg-precipitation according to BÁRÁNY to a constant level which is identical with both methods (16 μmoles/g protein). The adenine and phosphate content of the actin remains constant after the second purification procedure. Both procedures remove protein impurities present in the crude extract.

3. The protein impurities are, however, not removed by repeated isoelectric precipitation of the crude unpolymerized STRAUB-extract. This procedure removes only contaminating phosphate and nucleoside phosphate of the crude extract. The actin polymerizes spontaneously during isoelectric precipitation.

4. Ultracentrifugal sedimentation of F-actin, precipitation by $MgCl_2$ or isoelectric precipitation in presence of ATP do not inactivate the actin. The viscosity of F-actin, the ability for activating the ATP-ase of added L-myosin and the ATP-sensitivity of the resulting actomyosin remain constant even after repeating the purification procedure five times.

5. Repeated isoelectric precipitation of actin in absence of ATP leads to an increasing loss of adenine phosphate and also to a stepwise decrease of $Z\eta$ and ATP-sensitivity.

6. In the nucleoside phosphate of purified F-actin the proportion of adenine to phosphate is 1:2 as in ADP. Paperchromatographic methods reveal in addition traces of AMP and ATP.

7. G-actin and the contaminating proteins in the crude extracts contain also 16 μmoles adenine/g protein.

Abkürzungen: AMP, adenosin monophosphat; ADP, adenosin diphosphat; ATP, adenosin triphosphat.

8. From the content of adenosine phosphates bound to G- or F-actin (16 μmoles/g protein) the minimal molecular weight of the actin monomer is calculated as 62.000.

9. The proportion adenine: phosphate and paperchromatographic methods show, that in the crude unpolymerized extract the protein-bound nucleoside-phosphate consists of 70–75 % ATP and 25–30 % ADP. Only 70–75 % of the protein in the crude extract are able to polymerize.

10. However, G-actin obtained from purified F-actin containing also 70–75 % of its nucleoside phosphates as ATP does polymerize entirely. Thus whether or not ADP-G-actin polymerizes seems to depend on the history of the protein preparation.

11. G-actin, whose ability for polymerization has been destroyed by X-rays, nevertheless activates the L-myosin-ATP-ase to a normal extent. The same holds for actin partly denatured by isoelectric precipitation in the absence of ATP. Thus, the ability of actin for polymerisation and its ability to activate the L-myosin-ATP-ase are independent properties.

12. In phosphate or ATP containing solutions, purified F-actin binds ATP and inorganic phosphate reversibly (in addition to the tightly bound ADP). However, actin is not phosphorylated in presence of ATP.

EINLEITUNG

Durch die Entdeckung von H. HUXLEY und Mitarbeitern[1–4], dass die Kontraktion des Skelettmuskels auf einem Gleiten der Aktin-Filamente längs der L-Myosin-Filamente beruht, hat das Problem der Beteiligung des F-Aktins an der Kontraktion eine spezielle Form angenommen. So hat H. H. WEBER[5,6] in der schematischen Darstellung der chemischen Theorie der Filament-Verschiebung das Aktin als P-Akzeptor für das γ-Phosphat des ATP gewählt. Diese Wahl ist zwar mehr oder minder willkürlich, auf der anderen Seite aber ist die Phosphorylierung des F-Aktin-ADP während der Depolymerisierung die einzige Phosphorylierungs-Reaktion, die an einem kontraktilen Protein bisher direkt nachgewiesen ist[7]. KOSHLAND[8] nimmt ähnlich wie WEBER an, dass die Kontraktion mit der Phosphorylierung eines kontraktilen Proteins beginnt, aber er führt Argumente dafür an, dass das γ-Phosphat des ATP bei der Spaltung auf das L-Myosin übertragen wird.

In dieser Situation ist es lohnend unser Wissen über die chemischen Beziehungen zwischen F-Aktin und organischen wie anorganischen Phosphaten zu überprüfen und zu erweitern.

Es scheint gesichert, dass F-Aktin das ADP als fest gebundene prosthetische Gruppe enthält[9–11]. Dagegen beruht die Meinung, das Aktin enthalte auch im G-Zustand ein Nukleosid-Phosphat fest gebunden, nur auf einer einzelnen Beobachtung von STRAUB *et al.*[12]. Diese Autoren beobachteten, dass nur ein Teil des ATP in polymerisationsfähigen G-Aktin-Lösungen durch Apyrase mit normaler Geschwindigkeit gespalten wird. Ebenso herrscht keine Einigkeit darüber, ob eine etwaige prosthetische Gruppe des G-Aktin aus ADP[13] oder ATP[12] besteht.

Um zu entscheiden, wie weit Phosphate überhaupt als prosthetische Gruppe an Aktin fest gebunden sind, muss festgestellt werden, ob Aktin ganz bestimmte Mengen an Nukleosid-Phosphaten und anderen Phosphaten auch dann enthält, wenn durch extreme Reinigung alle Phosphate aus dem Lösungsmittel entfernt werden.

TABELLE I

NUKLEOSID-GEHALT VON AKTINEN VERSCHIEDENER HERSTELLUNGSART

	Präparat	*Reinigungsverfahren*	*Nukleosid-Gehalt in µMol/g Eiweiss*			*Funktionsproben des Aktins*
			Gesamt	*ATP*	*ADP*	
	F-Aktine					
PERRY[11]	Myofibrillen	6-malige Waschung mit 0.08 *M* Borat	20.2 pro g F-Aktin in der Fibrille	2.3	13.5	Keine
HASSELBACH[10]	Fibrillenschatten*	6-malige Waschung in 0.1 *M* KCl	18.0 pro g F-Aktin in den Fibrillenschatten	1.1	16.9	Keine
SZENT-GYÖRGYI[14]	H_2O-Extrakt aus Aceton-Trockenpulver, polymerisiert	ungereinigt	15.1	2.3	11.6	Keine
MOMMAERTS[9]	H_2O-Extrakt aus Aceton-Trockenpulver, polymerisiert	1-malige Waschung auf der Ultrazentrifuge	15–17			Keine
Diese Arbeit	H_2O-Extrakt aus Aceton-Trockenpulver, polymerisiert	4-malige Waschung auf der Ultrazentrifuge	16.0 Konstant nach d. 2. Waschung	Spuren	~15.0	Zη der Aktinlösung ATP-Empfindlichkeit der L-Myosin-Aktin-Mischung Aktivierung von L-Myosin-ATPase
		4-malige $MgCl_2$-Fällung	17.0 Konstant nach 2. Fällg.			
	Polymerisiert durch isoelektrische Fällung	5-malige Fällung** in ATP-Gegenwart	14.0 über alle Fällungen konstant			
	G-Aktine					
STRAUB UND FEUER[12]	H_2O-Extrakt aus Aceton-Trockenpulver	ungereinigt		12–19		Keine
SZENT-GYÖRGYI[14]	H_2O-Extrakt aus Aceton-Trockenpulver	ungereinigt	11.8	7.7	1.9	Keine
Diese Arbeit	H_2O-Extrakt aus Aceton-Trockenpulver	3-malige Behandlung mit Amberlite IRA 400	17.0 von d. 1. zur 3. Behandlung konstant	~13.0	~4.0	Nach Polymerisierung Tests wie bei F-Aktin
	Mittelwert für G- und F-Aktin dieser Arbeit		16.0			
	Mittelwert über G- und F-Aktine aller Autoren		16.0			

* Fibrillenschatten = fibril ghosts = Fibrillen nach Extraktion des L-Myosins.

** Isoelektrische Fällung in Abwesenheit von ATP führt zu fortlaufender Abnahme des gebundenen Nukleosids.

Eine praktisch vollständige Abwesenheit freier Phosphate scheint bisher nur in den Arbeiten von HASSELBACH[10] vorzuliegen, der seine Aktin-Präparationen aus 8-mal gewaschenen Fibrillen hergestellt hat.

Da Aktin sehr leicht denaturiert, muss geprüft werden, ob mit fortschreitender Reinigung der Phosphat-Anteil des Aktins fortlaufend abnimmt oder aber ein Endwert erreicht wird, der durch weitere Reinigungsprozeduren gleicher Art nicht mehr vermindert wird. Diese Prüfung scheint bisher nicht vorgenommen worden zu sein (Tabelle I).

Die Nukleosid-Phosphat-Bestimmungen in G-Aktin-Lösungen scheinen überhaupt nur an ungereinigten STRAUB'schen Rohextrakten durchgeführt worden zu sein.

Es werden infolgedessen in der vorliegenden Arbeit eine Anzahl von Reinigungsverfahren darauf geprüft, ob sie zu konstanten Endwerten an Phosphaten im G- und F-Aktin führen und die so gefundenen Werte an Phosphat und Nukleosid-Phosphat mit den Angaben der Literatur verglichen. Es wird ferner geprüft, wie weit die Reinigungsverfahren die kolloidalen und funktionellen Eigenschaften des F-Aktin verändern.

I

Die Verunreinigung eines Rohextraktes aus Aceton-Trockenpulver nach STRAUB hängt sehr stark von der Herstellung des Trockenpulvers ab. Wenn der Muskelbrei durch ausgiebige Waschung vollständig von Salzen befreit ist und hinterher durch gründliche Aceton-Waschung schnell völlige Trocknung erreicht wird, enthält der Rohextrakt neben polymerisationsfähigem Aktin nur etwa 20–30 % an langsam sedimentierenden Eiweiss-Verunreinigungen. Ein solcher Extrakt enthält ausserdem nicht

TABELLE II

VERTEILUNG VON NUKLEOSID UND PHOSPHAT VOR UND NACH ULTRAZENTRIFUGATION VON STRAUB'SCHEN AKTIN-EXTRAKTEN

	mg Eiweiss/ml	*Nukleoside μMol/ml spektrophotometrisch A = Adenosin I = Inosin*	*Phosphate in μMol/ml*			
				Anorganisches Phosphat		
			Nukleosid-phosphat	*Im Perchlorsäure Überstand*	*In der Perchlorsäurefällung*	*Gesamt-Phosphat*
(a) Rohextrakt nach Polymerisation	3.02	0.145[*] A I 0.067 0.079	0.164	0.253[**]	0.045	0.462
Nach Ultrazentrifugation:						
(b) Überstand	0.74	0.100[*] A I 0.022 0.078	0.074	0.224[**]	0.017	0.315
(c) Aktin	2.28	0.045 A I 0.045 —	0.09	0.029	0.032	0.151

[*] Für das abzentrifugierte Aktin ergibt sich die Absorptionskurve des Adenins; die Menge ist aus der Absorption bei 260 $m\mu$ mit dem Absorptionskoeffizienten des Adenosins berechnet. Im Überstand und Rohextrakt liegt das Absorptionsmaximum bei 250 $m\mu$. Es handelt sich möglicherweise um eine Mischung von Adenosin- und Inosin-Derivaten. Die Nukleosid-Menge wurde aus der Absorption bei 250, 260 und 265 $m\mu$ nach dem Verfahren von BENDALL UND DAVEY[26] berechnet und der Adenosin- und Inosin-Anteil ermittelt.

[**] ~ 0.045 μMol anorganisches Phosphat sind bei der Polymerisation freigesetzt worden.

unbeträchtliche Mengen an anorganischem Phosphat und geringere Mengen an Nukleosid-Phosphat, die nicht an das Aktin gebunden sind.

Diese Menge hängt von der Extraktionsdauer ab. Bei einer Extraktionszeit von 2 Min entfallen etwa 200 μMol solchen Phosphats auf 1 g Protein, bei einer Extraktionsdauer von 30 Min nur etwa 100 μMol. Die Abhängigkeit der Proportion Phosphat: Protein von der Extraktionsdauer beruht darauf, dass die Verunreinigungen schnell und das Protein langsam extrahiert werden. Die Verteilung der Phosphate und des Eiweisses nach einer Extraktionsdauer von 30 Min ergibt sich aus Tabelle II.

Die Reinigung des polymerisationsfähigen Aktins von anderen Proteinen ist spezifisch, (a) wenn das Aktin polymerisiert und auf der Ultrazentrifuge sedimentiert wird, (b) wenn das F-Aktin durch niedrige Konzentrationen von $MgCl_2$ nach BÁRÁNY[15] umgefällt wird. Eine sehr viel geringere Spezifität ist zu erwarten bei der isoelektrischen Fällung[12], obwohl auch wiederholte isoelektrische Fällung verhältnismässig häufig als Reinigungsverfahren verwendet wird. Es werden in erster Linie diese 3 Reiningungsverfahren benutzt und geprüft.

Die Entfernung ausschliesslich der organischen und anorganischen Phosphat-Verunreinigungen ist auch mit anderen Methoden (Ionenaustauscher, Dialyse) möglich.

Der Fortschritt in der Reinigung des Aktins von den anderen Eiweisskörpern wird kontrolliert durch das Anwachsen der Viskositätszahl ($Z\eta$[*]) der F-Aktin-Lösungen und der ATP-Empfindlichkeit[*] der Mischungen des F-Aktin-Präparates mit L-Myosin-Lösungen. Die Aktivierung der L-Myosin-ATPase durch die Aktin-Präparate nimmt mit steigender Reinheit des Aktin nicht zu.

II

Wird das Aktin im Rohextrakt polymerisiert und anschliessend 4 mal mit der Ultrazentrifuge oder durch $MgCl_2$ gefällt und resuspendiert (siehe TECHNISCHER TEIL), so wird der Gesamtgehalt an Phosphat pro Gramm Protein, das durch Perchlorsäure freigesetzt wird, mit der dritten Umfällung konstant; das gleiche gilt für den sehr niedrigen Gehalt an anorganischem durch Perchlorsäure ablösbaren Phosphor (Fig. 1 und 2). Der Adeningehalt fällt schon von der ersten zur zweiten Umfällung nur sehr wenig ab und bleibt dann konstant, weil der Rohextrakt mit Adenin viel weniger verunreinigt ist als mit anorganischem Phosphat.

Der optisch bestimmte Nukleotid-Gehalt stimmt in allen Versuchen exakt überein mit dem Nukleotid-Gehalt, der sich aus der Differenz von Gesamtphosphat und anorganischem Phosphat ergibt, wenn das Nukleotid als Di-phosphat angesehen wird. Alle Phosphat-Werte bei Reinigung des F-Aktins auf der Ultrazentrifuge und durch $MgCl_2$-Fällung sind identisch. Sie betragen nach vollständiger Entfernung der Phosphat-Verunreinigungen im Mittel 16 μMol ADP/g Protein und 3 μMol anorganisches Phosphat/g Protein als durch Perchlorsäure ablösbare prosthetische Gruppen.

Die Eiweissverunreinigungen des Rohextraktes sind offensichtlich bereits durch die erste Umfällung sowohl im Ultrazentrifugen- wie im $MgCl_2$-Verfahren praktisch vollständig beseitigt. Dies geht aus den Kurven von $Z\eta$ und ATP-Empfindlichkeit in der oberen Hälfte der Fig. 1 und 2 hervor. Die Ordinate dieser Kurven gibt die Viskositätszahl und die ATP-Empfindlichkeit der Eiweisspräparate der verschiedenen Reinigungsstufen, bezogen auf den Gehalt an Protein (nicht an Aktin). Infolgedessen müssen Viskositätszahl und ATP-Empfindlichkeit mit fortschreitender Reinigung des Aktins ansteigen. Zahlenmässig sind beide Werte ausgedrückt in % des Standardwertes. Der Standardwert ist der Generalmittelwert zahlreicher gereinigter Aktin-Präparate. Er beträgt für $Z\eta$ 0.85 bei dem hier gegebenen sehr niedrigen Geschwindigkeits-Gradienten von 250 sec^{-1}. Der Standardwert der ATP-Empfindlichkeit beträgt unter den Bedingungen dieser Arbeit 120 % (siehe TECHNISCHER TEIL).

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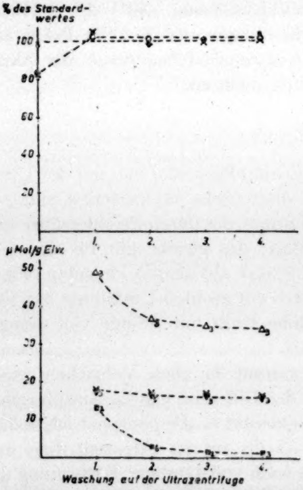
[*] ATP-Empfindlichkeit $= \frac{Z\eta - Z\eta_{ATP}}{Z\eta_{ATP}} \cdot 100$, wobei $Z\eta = \frac{2.3 \cdot \log \eta_{rel}}{c}$ ($Z\eta = 0.1$ intrinsic viscosity $[\eta]$) (vergl. PORTZEHL *et al.*[24]). Da die Arrhenius Beziehung für F-Aktin-Präparate nicht streng gilt, wurden alle Messungen bei einer Eiweiss-Konzentration ausgeführt.

Mit beiden Methoden ist dieser Höchstwert bereits nach der ersten Umfällung erreicht, obwohl im Rohextrakt mit seinen Eiweissverunreinigungen diese Werte nur zwischen 80 und 90 % des Standardwertes für gereinigte Aktine liegen.

Wir können daher die oben angegebenen Werte für die prosthetische Gruppe auf reines Aktin beziehen. Infolgedessen ergibt sich aus dem Gehalt an ADP ein Äquivalent-Gewicht des Aktins von 62,000. Dieser Wert liegt im Streubereich der bisherigen Molekulargewichtsbestimmungen für das G-Aktin-Monomer[9, 16–20, 28]. Diesem Wert kommen auch die schon früher gefundenen Werte für den ADP-Gehalt



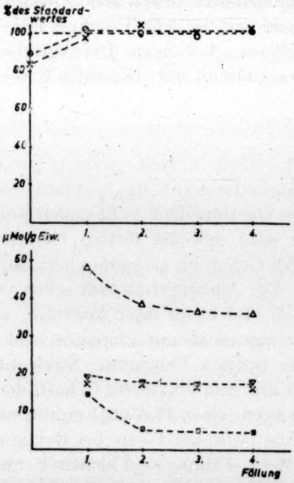


Fig. 1. Reinigung von F-Aktin durch Waschung auf der Ultrazentrifuge. Abszisse: Zahl der Waschungen. Ordinate: Untere Hälfte durch Perchlorsäure ablösbare Phosphat-Fraktionen in µMol/g Eiweiss. △---△, Gesamt-Phosphat; ■---■, anorganisches Phosphat; ×---×, ½ Differenz zwischen Gesamt-Phosphat und anorganischem Phosphat; ▼---▼, Adenin (spektrophotometrisch). Die Zahlen an den Symbolen bedeuten die Anzahl der Versuche. Obere Hälfte: $Z\eta$, ATP-Empfindlichkeit, ATPase-Aktivierung in % der Standardwerte. ○---○, $Z\eta$ bei 0.1 *M* KCl, 0.001 *M* $MgCl_2$, 0.001 *M* Histidin-Puffer; ×---×, ATP-Empfindlichkeit in 0.6 *M* KCl; ●---●, Aktivierung der L-Myosin-ATPase bei 0.085 µ. Mittelwerte aus 2–4 Versuchen. pH 7.0. Temperatur, 20°.

Fig. 2. Reinigung des F-Aktins durch Umfällung mit $MgCl_2$. Abszisse: Zahl der Umfällungen, die den Einzelmessungen vorhergegangen sind. Sonst wie Fig. 1. Mittelwerte aus 2 Versuchen.

des F-Aktins sehr nahe (siehe Tabelle I). Die Übereinstimmung beweist, dass auch die früheren ADP- und Adenin-Analysen sich auf weitgehend reines F-Aktin beziehen, obwohl die Reinigung dort nicht systematisch bis zur Konstanz des gebundenen ADP fortgeführt wurde. Die Übereinstimmung ist nicht erstaunlich, weil die vorliegenden Untersuchungen zeigen, dass die Verunreinigungen bereits bei der ersten Umfällung fast vollständig verschwinden (mit Ausnahme des anorganischen Phosphates).

III

Es ist nicht möglich, das Aktin des Rohextraktes zu polymerisieren und das so gewonnene F-Aktin durch isoelektrische Umfällung nach STRAUB zu reinigen, weil es nicht möglich ist, den isoelektrischen F-Aktin-Niederschlag wieder klar zu lösen. Wird dagegen das G-Aktin des Rohextraktes ohne vorherige Polymerisation isoelektrisch gefällt, so kann der Niederschlag bei pH 7 wieder aufgelöst werden.

Das ist deshalb sehr merkwürdig, weil dieser Niederschlag nach seiner Auflösung sich viskosimetrisch mehr oder minder wie F-Aktin verhält. Bereits nach der ersten Umfällung besitzt die neutralisierte Lösung ohne Zusatz polymerisierender Salze etwa 75 % der Viskosität, die durch nachträglichen Salzzusatz erreicht werden kann. Nach der dritten Umfällung ist die Viskosität ohne Salzzusatz auf Werte angestiegen, die durch den Zusatz polymerisierender Salze nicht mehr gesteigert werden können (Tabelle III). Dieser Befund sieht so aus, als ob die kleinen Mengen an Acetat-Puffer und Bikarbonat, die für die pH-Verschiebungen gebraucht werden, bereits für eine "normale" Polymerisation genügen. Die Reversibilität der isoelektrischen Fällung des spontan entstandenen F-Aktin muss infolgedessen auf die Abwesenheit von KCl, die Irreversibilität der Fällung des normal gebildeten F-Aktin auf die Anwesenheit von 0.1 *M* KCl bezogen werden.

TABELLE III

POLYMERISIERUNG DES G-AKTINS DURCH ISOELEKTRISCHE FÄLLUNG

(in Gegenwart von $2 \cdot 10^{-4}$ *M* ATP.)

	Acetat-Konzentration	*Zη nach isoelektrischer Fällung in % von Zη nach Salzzusatz*				
1. Fällung	0.002–0.007	78.5	71.6	72.0	58.5	100
2. Fällung	0.014–0.02	97.8	104	88	75.5	111
3. Fällung	0.017–0.05	108	100	110	88.0	110
4. Fällung	0.035–0.1	105	104	114	96.0	114
5. Fällung	0.045–0.15	111	103	97		115

Wird nun das Aktin der isoelektrischen Fällung als F-Aktin angesehen und in Abwesenheit von ATP mehrfach isoelektrisch umgefällt (Fig. 3), so zeigt sich, dass das Aktin fortschreitend denaturiert wird. Die Menge des gebundenen Nukleosid-Phosphates nimmt dabei fortlaufend ab. Nach der 6. Umfällung ist der ADP-Gehalt auf weniger als die Hälfte des Normalwertes abgesunken. Ebenso wird der Polymerisationsgrad von Fällung zu Fällung kleiner (abgelesen an der Abnahme von Zη und ATP-Empfindlichkeit), trotz nachträglichen Zusatzes optimaler KCl- und $MgCl_2$-Mengen. Dagegen bleibt die ATPase-Aktivität des künstlichen Aktomyosins, das aus diesen nachpolymerisierten Aktin-Präparaten hergestellt wird, völlig normal.

Die isoelektrischen Umfällungen wirken nicht denaturierend, wenn sie in Gegenwart von $2 \cdot 10^{-4}\,M$ ATP (siehe [12]) vorgenommen werden (Fig. 4). Die Aktivierung der L-Myosin-ATPase, die ATP-Empfindlichkeit und die Viskositätszahl ändern sich über 5 Umfällungen nicht (das Aktin-Präparat ist vor der viskosimetrischen und ATPase-Testung durch Salz polymerisiert oder nachpolymerisiert).

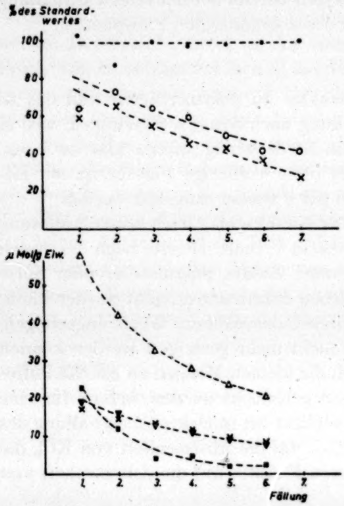


Fig. 3. Reinigung des Aktins durch isoelektrische Fällungen in Abwesenheit von ATP. Abszisse: Zahl der isoelektrischen Fällungen. Sonst wie Fig. 1 und 2. Mittelwerte aus 2–3 Versuchen.

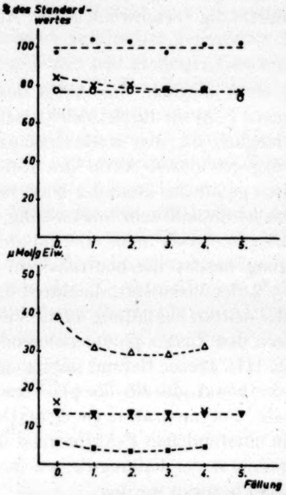


Fig. 4. Reinigung des Aktins durch isoelektrische Fällungen in Gegenwart von ATP. Abszisse: Zahl der isoelektrischen Fällungen. Zeichenerklärung wie Fig. 1 und folgende.

Die Bestimmung der Phosphat-Fraktionen hat nur Sinn, wenn das zugesetzte ATP und damit gleichzeitig auch alle P-Verunreinigungen im Rohextrakt und nach jeder Umfällung vor der Analyse durch einen Ionen-Austauscher beseitigt werden (siehe TECHNISCHER TEIL). Unter diesen Umständen sind der Gehalt an gebundenem Adenin und gebundenem anorganischem Phosphat vom Rohextrakt bis zur 5. Umfällung konstant. Nur der Gehalt an Gesamt-Phosphat nimmt durch die erste Umfällung beträchtlich ab, weil durch die gleichzeitige Polymerisation aus Aktingebundenem ATP Aktingebundenes ADP wird*.

Man kann auf Grund der beobachteten Viskositätszunahme bei isoelektrischer Fällung und der gleichzeitigen Phosphat-Abspaltung, die mit der Polymerisierung verbunden ist, annehmen, dass es sich auch bei dieser Art der Polymerisierung um den normalen Mechanismus des Überganges von G-Aktin zu F-Aktin handelt. Wenn man das annimmt, ist zu folgern, dass nicht nur G-Aktin, sondern auch F-Aktin bei pH 4.8 immer dann denaturiert, wenn kein ATP zugegen ist.

Die Ergebnisse, die in Fig. 4 dargestellt sind, zeigen, dass die isoelektrische

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* Dass bei der Ansäuerung auf pH 4.8 eines Austauscherbehandelten G-Aktin-haltigen Rohextraktes gebundenes Phosphat abgespalten wird, ist auch durch direkte Analyse des freigesetzten Phosphates gesichert.

Fällung auch in Gegenwart von ATP nicht als Reinigungsverfahren des Aktins empfohlen werden kann. Denn die ATP-Empfindlichkeit und die Viskositätszahl bleiben während der Reinigungsschritte auf dem niedrigen Wert stehen, der bereits im Rohextrakt gefunden wird (ähnlich 80% des Wertes einer gereinigten Aktin-Lösung, vergl. Fig. 4). Die verunreinigenden Eiweiss-Körper des Rohextraktes werden offenbar nicht entfernt. Es sieht also so aus, als wenn das isoelektrische Reinigungsverfahren nicht viel anderes leistet als Behandlung mit Ionenaustauschern oder Dialyse. Denn auch Dialyse und Behandlung mit geeigneten Ionenaustauschern beseitigen die Verunreinigungen an organischen und anorganischen Phosphaten, ohne gebundene Phosphate aus der Bindung an das Eiweiss abzuspalten. Für den Ionenaustauscher ergibt sich das aus den Erfahrungen bei der isoelektrischen Fällung (siehe auch Abschnitt IV), für die Dialyse aus Fig. 5.

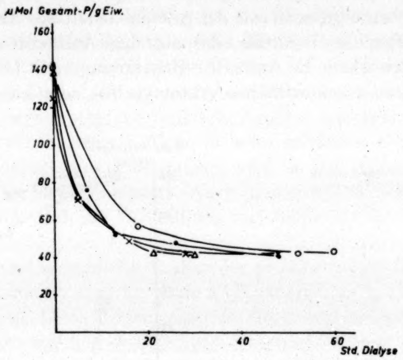

Fig. 5. Änderungen des Phosphat-Gehaltes von polymerisierten STRAUB-Extrakten während der Dialyse. Ordinate: Gesamt-Phosphat des Extraktes in µMol/g Eiweiss. Abszisse: Dauer der Dialyse. Jede Kurve repräsentiert einen Einzelversuch. Dialyse gegen 0.1 *M* KCl, 0.001 *M* $MgCl_2$, 0.001 *M* Histidin-Puffer. pH 7.0. Temperatur, 0°. Alle 3 Stunden Wechsel der Dialysierflüssigkeit. Dialysierschlauch der Firma Kalle und Co., Wiesbaden-Biebrich.

IV

Es wird im allgemeinen angenommen[7,12,20], dass der Adenin-Gehalt von G-Aktin und F-Aktin gleich ist und dass die Polymerisation verbunden ist mit der Reaktion

$$n\,(\text{G-Aktin-ATP}) \underset{\substack{+\text{ ATP oder CP-System}\\ -\text{ Salz}}}{\overset{+\text{ Salz}}{\rightleftharpoons}} \text{F-Aktin-ADP(I)} + \text{n-Phosphat}$$

Experimentelle Zahlenangaben über den Adenin-Gehalt des G-Aktins sind selten und widerspruchsvoll. STRAUB und Mitarbeiter[12] geben an, dass Rohextrakte 11–19 µMol an Aktin-gebundenem ATP enthalten, A. G. SZENT-GYÖRGYI findet im Rohextrakt ~ 7 µMol/g Eiweiss[14]. GERGELY[13] schliesst aus seinen Versuchen, dass G-Aktin, das durch Depolymerisation von F-Aktin in Anwesenheit von ATP entstanden ist, nicht ATP, sondern ADP enthielte. STROHMAN[7] findet dagegen nach Depolymerisation von F-Aktin in Gegenwart von Phosphokreatin und Phosphokinase ein ATP-haltiges G-Aktin.

Werden durch Amberlite IRA 400 (100 mg/ml) alle kristalloiden Ionen des Rohextraktes entfernt, so ergeben sich 16–18 µMol Adenin/g Eiweiss (Fig. 6). Dieser Adenin-Gehalt ändert sich auch bei weiteren Behandlungen mit Amberlite nicht mehr. Der Adenin-Gehalt des Proteins im Rohextrakt ist also dem Adenin-Gehalt des F-Aktins gleich. Da im Mittel nur etwa 75 % des Proteins im Rohextrakt polymerisierbar sind, bedeutet diese Feststellung, dass der Adenin-Gehalt des nicht polymerisierbaren Proteins ebenso gross ist wie der Adenin-Gehalt des Aktins.

Die Eigenschaften des F-Aktins, das aus dem Amberlite-gereinigten G-Aktin hergestellt ist, werden durch die Amberlite-Behandlung nicht (ATP-Empfindlichkeit und ATPase-Aktivität des künstlichen Aktomyosins) oder kaum (Viskositätszahl) verändert (Fig. 6).

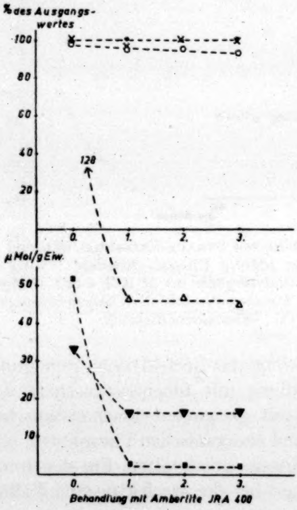

Fig. 6. Die Wirkung von Amberlite IRA 400 auf die Eigenschaften der Proteine im unpolymerisierten Straub'schen Rohextrakt. Abszisse: Zahl der Behandlungen vor der Messung. 1 und 2, Behandlung je 100 mg Amberlite/ml; 3, Behandlung 150 mg Amberlite/ml. Ordinate und Zeichengebung wie in Fig. 1–4. Vor den Funktionsproben der oberen Hälfte der Abbildung wurde das G-Aktin polymerisiert. Mittelwerte aus 2 Versuchen.

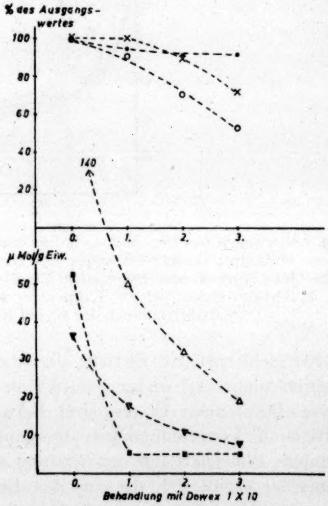

Fig. 7. Einfluss der Behandlung von unpolymerisiertem Straub-Extrakt mit Dowex 1 × 10. Abszisse: Zahl der Behandlungen mit Dowex, 1. Behandlung 160 mg/ml, 2. Behandlung 230 mg/ml, 3. Behandlung 400 mg/ml. Sonst wie Fig. 6.

Dagegen führt Behandlung mit grösseren Quantitäten des feinkörnigen Anionen-Austauschers Dowex 1 × 10 zu einer Denaturierung, die mit der Zahl der Dowex-Inkubierungen zunimmt (Fig. 7).

Aus der Differenz zwischen dem Gesamt-Phosphat und dem anorganischen Phosphat, das durch Säure ablösbar ist, ergibt sich, dass auf 1 g Protein-gebundenes Adenin des gereinigten Rohextraktes 2.7 Phosphat-Reste entfallen. Das entspricht einer Proportion von 0.7 ATP zu 0.3 ADP. Auch die papierchromatographische

Analyse zeigt viel ATP neben wenig ADP. Während der Polymerisation werden auf 4 Teile Adenin 3 Teile Phosphat abgespalten (Mittel aus 10 Versuchen). Also wird alles ATP während der Polymerisation gespalten. Dieser Befund passt zu der Tatsache, dass sich durchschnittlich 75 % des Eiweisses auf der Ultrazentrifuge sedimentieren lassen. Dass entsprechend 75 % des Nukleosid-Gehaltes Phosphat abgespalten und gleichzeitig 75 % des Proteins polymerisiert werden, spricht dafür, dass ausschliesslich das ATP–G-Aktin in F-Aktin–ADP übergeht. Das unsedimentierbare und offensichtlich auch unpolymerisierbare Protein enthält die restlichen 25 % des Adenins zum grössten Teil als ADP (21 % des Gesamt-Adenins), zum kleinen Teil als AMP (4 % des Gesamt-Adenins). Dies ergibt sich rechnerisch aus dem Verhältnis Adenin: Phosphat und ist durch papierchromatographische Analyse gesichert.

Wenn ein abzentrifugiertes F-Aktin in einer salzfreien $1 \cdot 10^{-4}$ *M* ATP-Lösung mit Hilfe des Homogenisators resuspendiert wird, so fällt seine Viskositätszahl von 0.85 auf 0.05, d.h. es depolymerisiert. Auch in solchen *in vitro* depolymerisierten G-Aktinen findet sich nach der Behandlung mit Amberlite ein Gehalt von 16 μMol Adenin/g Aktin[30].

Die säulenchromatographische Analyse der prosthetischen Gruppe des G-Aktins ergibt, dass das Adenin zu etwa 70 % als ATP, zu etwa 20 % als ADP und zu etwa 10 % als AMP vorliegt. Diese Zahlen zeigen im Gegensatz zu GERGELY's Ergebnissen, dass nicht nur in Anwesenheit des Phospho-Kreatin-Systems[7], sondern auch in Anwesenheit von ATP während der Depolymerisation die prosthetische Gruppe ADP weitgehend durch ATP ersetzt wird. Der Gehalt des Proteins an Adenosin-Phosphat ist im Rohextrakt und nach Depolymerisierung gleich, die Verteilung der prosthetischen Gruppe auf die Formen ATP, ADP und AMP ist sehr ähnlich. Das G-Aktin, das durch Depolymerisierung von F-Aktin gewonnen ist, polymerisiert aber im Gegensatz zum Rohaktin praktisch vollständig. Das zeigt, dass es auch polymerisationsfähiges ADP-G-Aktin gibt. Die Bedingungen, unter denen sich polymerisationsfähiges ADP-G-Aktin bildet, werden in einer späteren Mitteilung behandelt[31].

V

Es wird vielfach angenommen, dass das nicht polymerisierbare Protein des STRAUB'schen Rohextraktes nichts anderes sei als denaturiertes Aktin. Die bisher fehlenden Argumente für diese Annahme können aus den Versuchen dieser Arbeit abgelesen werden.

1. Fig. 4 und Fig. 6 zeigen, dass an 1 g Protein des Rohextraktes die gleiche Adenin-Menge von 15–16 μMol gebunden ist, die auch im gereinigten Aktin-Präparat gefunden wird.
2. Fig. 1 und Fig. 2 zeigen, dass auch die Aktivierung der L-Myosin-ATPase durch 1 g Protein des Rohextraktes gleich ist der Aktivierung durch 1 g gereinigtes Aktin. Aktivierung der L-Myosin-ATPase und der Adenin-Gehalt sind Eigenschaften, die für Aktin sehr spezifisch sind. Trotzdem sind die nicht polymerisierbaren Proteine des Rohextraktes in diesen beiden Eigenschaften dem gereinigten Aktin gleich oder sehr ähnlich.

Auf der anderen Seite zeigt unsere Arbeit (Fig. 3 und Fig. 7), dass die artefizielle Denaturierung des Aktins seine Fähigkeit der ATPase-Aktivierung selbst dann unberührt lässt, wenn ATP-Empfindlichkeit, Viskositätszahl und Adenin-Gehalt stark abnehmen. Es ist sogar möglich, durch Röntgenbestrahlung von G-Aktin die Polymerisationsfähigkeit stark herabzusetzen und gleichzeitig die Aktivierung der L-Myosin-ATPase durch das röntgengeschädigte, unvollständig polymerisierte Aktin bis zu 50 % zu erhöhen[21] (Fig. 8). Durch sehr hohe Röntgendosen lässt sich sogar die Polymerisationsfähigkeit des G-Aktins fast vollständig aufheben, ohne dass die Aktivierung der L-Myosin-ATPase unter den Normalwert sinkt (Fig. 8).

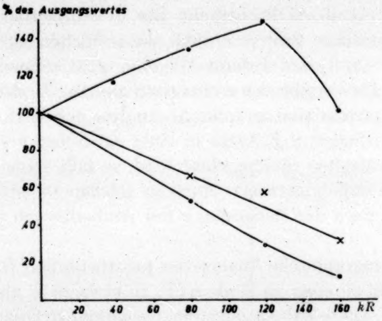

Fig. 8. Einfluss von Röntgenstrahlung auf die Reaktionsfähigkeit von G-Aktin im STRAUB'schen Rohextrakt. Abszisse: Röntgendosis. Ordinate: $Z\eta$, ATP-Empfindlichkeit und ATPase-Aktivierung in % der unbestrahlten Probe. Zeichen wie in Fig. 1–4. Dosisleistung 1500 R/min, 2 mm Al-Filter, 250 kV, 15 mA, 4.3 mg Eiweiss/ml; pH, 7.0; Temperatur, 20°.

Diese Beobachtungen legen folgende Schlüsse nahe: Denaturierung des Aktins beeinträchtigt in erster Linie die Polymerisationsfähigkeit und ihre Folgeerscheinungen, d.h. die ATP-Empfindlichkeit des künstlichen Aktomyosins. Für diese Wirkung scheint es zu genügen, dass das gebundene ATP zu ADP oder AMP wird (vergl. Abschnitt IV). Eine weitergehende Denaturierung führt zusätzlich zum Verlust prosthetischer Gruppen, ohne gleichzeitigen Verlust der Aktivierungsfähigkeit für L-Myosin-ATPase. Also scheinen die prosthetischen Gruppen keine Bedeutung für die enzymatische Interaktion von Aktin und L-Myosin zu besitzen.

ANHANG

Ausser den anorganischen und Adenosin-Phosphaten, die von G- und F-Aktin als prosthetische Gruppe fest gebunden sind, finden sich am F-Aktin auch organische und anorganische Phosphate in reversibler Gleichgewichtsbindung.

Wenn F-Aktin aus Lösungen mit einem abgestuften, nicht zu hohen Gehalt von anorganischem ^{32}P oder $[^{32}P]$ATP mit der Ultrazentrifuge abgetrennt wird, so ist der Gehalt des Niederschlages an ATP, anorganischem Phosphat und Radioaktivität grösser als der Gehalt des Überstandes. Wir beziehen den Überschuss an Phosphaten und Radioaktivität, der im Niederschlag gefunden wird, auf das g F-Aktin im Niederschlag, wir bezeichnen das Resultat als scheinbar gebundenes Phosphat, ATP oder Radioaktivität und messen diese Beträge in μMol/g F-Aktin. Die scheinbar gebundenen Mengen ergeben sich in ihrer Abhängigkeit von der zugegebenen Konzentration an anorganischem Phosphat bezw. ATP aus Fig. 9.

Die Fig. 9 zeigt, dass die scheinbar gebundenen Mengen der Phosphate mit wachsender Konzentration zunächst zunehmen und für das ATP wieder abnehmen und schliesslich sogar negativ werden, sobald die Konzentration von $1 \cdot 10^{-3}$ überschritten ist. Es ist zu erwarten, dass die scheinbar gebundene Menge schliesslich wieder abnimmt, weil der Überschuss des Reaktionspartners im eiweisshaltigen Volumen des Systems, d.h. in diesem Falle im Pellet nicht nur von der wirklich gebundenen Menge des Reaktionspartners abhängt, sondern auch von dem nichtlösenden Raum, der durch das Eigenvolumen des Eiweisses und seines Hydratationsmantels bedingt ist. Der nichtlösende Raum aber hat eine negative scheinbare Bindung zur Folge. Sein Einfluss wächst mit zunehmender Konzentration des zugegebenen Phosphats bezw. ATP und wird mehr und mehr vorherrschend, sobald die wahre Bindung den Sättigungswert erreicht hat. Solange der nichtlösende Raum des F-Aktins für Phosphat und ATP unbekannt ist, ist es nicht möglich, die wahre Bindung der Reagenzien aus den Kurven der scheinbaren Bindung abzuleiten. Dass die scheinbare Bindung für ATP viel früher abnimmt als für Phosphat, kann z.B. bedeuten, dass der nichtlösende Raum für das ATP-Ion grösser ist als für das anorganische Phosphat-Ion; es kann aber auch bedeuten, dass die Sättigung des Aktins mit ATP viel früher erreicht wird als mit Phosphat. Die Kurven zeigen also nur, dass eine reversible Bindung zusätzlich zur festen Bindung vorhanden ist.

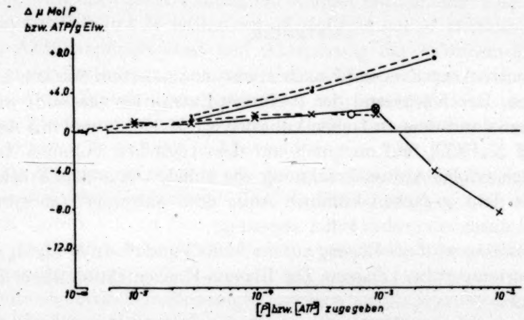

Fig. 9. Scheinbare Bindung von ortho-Phosphat und ATP durch gereinigtes F-Aktin (vergl. METHODIK). Abszisse: Konzentration des zugegebenen anorganischen Phosphates bezw. ATP. Ordinate: nach oben positive scheinbare Bindung in μMol/g Eiweiss, nach unten negative scheinbare Bindung. Bindung von anorganischem Phosphat: ●—●, kolorimetrisch; ○---○, aus ^{32}P Aufnahme. ATP-Bindung: ×—×, $^1/_3$ des kolorimetrisch bestimmten Phosphat-Zuwachses; ×---×, $^1/_3$ des aus der Aktivitätsaufnahme bestimmten Phosphat-Zuwachses; ○---○, Adenin-Zuwachs.

Wichtiger ist Folgendes: die scheinbare Bindung von radiometrisch und analytisch bestimmbarem Phosphat ist immer gleich, sowohl für anorganisches Phosphat wie für ATP. Ausserdem beträgt für ATP die optisch bestimmte scheinbare Adenin-Bindung 1/3 der scheinbaren Bindung, die sich aus der Phosphat- und Aktivitätsbestimmung ergibt. Die Äquivalenz der scheinbaren Bindung von Phosphat, Radioaktivität und Adenin schliesst es aus, dass das F-Aktin neben der reversiblen ATP-Bindung auch noch Phosphat durch Transphosphorylierung in messbaren Mengen aufnimmt. Denn wenn γ-Phosphat des ATP auf das Aktin transphosphoryliert würde, müsste die scheinbare Bindung von Phosphat und Radioaktivität grösser sein als die scheinbare Bindung von Adenin.

Das Fehlen einer nachweisbaren Transphosphorylierung von ATP auf F-Aktin passt zu den früheren Befunden, durch die auf anderem Wege eine Transphosphorylierung von ATP auf F-Aktin aus Aceton-Trockenpulver ausgeschlossen wurde[8, 22].

METHODIK

Aktin: Acetontrockenpulver wird nach etwas modifiziertem STRAUB'schen Verfahren[17] gewonnen. Der Rückstand der L-Myosin-Extraktion (20 min) wird 1 mal mit 0.6 M KCl, 3 mal mit dem 10-fachen Volumen 0.1 M KCl, 2 mal mit Aqua dest., 1 mal mit 0.01 M $NaHCO_3$ und nochmals mit dem 10-fachen Volumen Aqua dest. gewaschen. Danach erfolgt Aceton-Trocknung wie üblich[23]. Aus dem Trockenpulver wird G-Aktin mit dem 30-fachen Volumen Aqua dest. extrahiert (30 min bei 0°). Der Extrakt wird durch ein grobes Filter abgesaugt.

Zur Polymerisation wird die Lösung auf 0.1 M KCl und 0.001 M $MgCl_2$ gebracht, 10 min bei Zimmertemperatur belassen. Die Eiweiss-Konzentration dieser Lösungen beträgt 3–4 mg/ml.

L-Myosin wird nach dem Verfahren von PORTZEHL[24] hergestellt. Die Lösungen werden in 50 % Glycerin bei —15° aufgehoben, das Glycerin vor Gebrauch durch Umfällung entfernt.

Aktinreinigungen

Durch Ultrazentrifugation: Der polymerisierte Rohextrakt wird 3 Stunden bei 80,000 × g (SPINCO Rotor 30) oder 1.5 Stunden bei 100,000 × g (SPINCO Rotor 40) bei 0° zentrifugiert. Man erhält einen festen glasigen Niederschlag von F-Aktin, der etwa 1/40 des ursprünglichen Volumens einnimmt. Das Zentrifugengläschen wird mit 0.1 M KCl ausgespült, das Aktin-Pellet wird mit einem Spatel in einen Glashomogenisator mit eingepasstem Teflon-Stempel (A. H. Thomas, Philadelphia, Best. Nr. 4288-B) gebracht und in 0.1 M KCl und 0.001 M $MgCl_2$ homogenisiert. Die Eiweiss-Konzentration wird wieder auf etwa 3 mg/ml gebracht. Es ist also möglich, den F-Aktin-Niederschlag direkt wieder in Lösung zu bringen, ohne den Umweg über eine Depolymerisation zu machen (vergl. Ref. 29). Diese Reinigung wird 4 mal vorgenommen. Bei der ersten Zentrifugation bleiben 20–30 % Eiweiss im Überstand, bei den nachfolgenden Zentrifugationen sedimentiert das Eiweiss jedesmal nahezu quantitativ.

$MgCl_2$-Fällung nach BÁRÁNY[15]: Das Aktin kann durch $MgCl_2$ von 0.025–0.1 M gefällt werden. Das Volumen des Niederschlages nimmt mit steigender $MgCl_2$-Konzentration ab (15 min zentrifugieren bei 15,000 × g). Der Niederschlag lässt sich leicht mit dem Teflon-Homogenisator aufrühren.

Zur mehrfachen Reinigung von F-Aktin benutzten wir 0.07 M $MgCl_2$. Das Aktin lässt sich dabei auf etwa 1/20 seines Volumens zusammenzentrifugieren. Bei der ersten Fällung werden 70–75 % des Proteins im Rohextrakt sedimentiert. In den nachfolgenden Reinigungsschritten lassen sich jeweils 85–90 % des erhaltenen Eiweisses abzentrifugieren. Die Aktin-Lösungen enthalten nach jedem Schritt etwa $5 \cdot 10^{-3}$ M $MgCl_2$.

Die isoelektrische Fällung[12]: Geschieht an G-Aktin. Mit wenig 1 M Acetat Puffer (pH 4.5) wird die G-Aktin-Lösung auf pH 4.8 gebracht, das gefällte Eiweiss abzentrifugiert (10 min 2,000 × *g*), der Niederschlag mit wenig $NaHCO_3$ unter Rühren bei pH ~ 7.0 wieder in Lösung gebracht. Wenn die Denaturierung des Aktins vermieden werden soll, ist $2 \cdot 10^{-4}$ *M* ATP während der ganzen Fällungs- und Wiederauflösungsprozeduren gegenwärtig. Für die Prüfung von $Z\eta$, ATP-Empfindlichkeit und ATPase-Aktivierung wird nach jeder Fällung ein Aliquot entnommen. Alle Aliquots werden gleichzeitig mit Salz (0.1 *M* KCl, 0.001 *M* $MgCl_2$, 0.001 *M* Histidin-Puffer) versetzt, bevor $Z\eta$, ATP-Empfindlichkeit und Aktivierung der L-Myosin-ATPase gemessen werden. Vor der Bestimmung von gebundenem Phosphat und gebundenem Nukleotid werden die Aktin-Proben (ohne Salzzusatz) mit Amberlite IRA 400 (100 mg/ml bei 2.5–3.0 mg Eiweiss/ml) 5 min gerührt und der Ionen-Austauscher dann abzentrifugiert. Geschieht die Fällung in Abwesenheit von ATP, tritt von der 3. Fällung an zunehmende Trübung auf, Zusatz von Ascorbinsäure[12] gewährt keinen Schutz.

Ionenaustauscher-Behandlung: Die G-Aktin-Lösungen (~ 3 mg Eiweiss/ml) werden mit Dowex 1 × 10 (200–400 mesh, Chlorid-Form) oder Amberlite IRA 400 (20–50 mesh, Chlorid- oder Acetat-Form) 5 min bei pH 7 gerührt, dann über ein grobes Filter abgesaugt.

Die viskosimetrischen Messungen erfolgen in Kapillar-Viskosimetern mit gewundener Kapillare (Höhendifferenz 12 cm, Kapillarlänge ~ 70 cm, Kapillardurchmesser 0.9 mm, Durchlauf-Volumen ~ 1 ml, Auslaufzeit für H_2O ~ 25 sec) bei 20° und pH 7. Zur Bestimmung der Viskositätszahl beträgt die Eiweiss-Konzentration 0.7 mg/ml. Die Kapillaren haben ein Gefälle von ~ 250 sec^{-1}, für gereinigte Aktine ergeben sich $Z\eta$-Werte von ~ 0.85 (0.1 *M* KCl, 0.001 *M* $MgCl_2$, 0.001 *M* Histidin-Puffer).

Zur Bestimmung der ATP-Empfindlichkeit[24] werden künstliche Aktomyosin-Sole hergestellt, bei denen das Verhältnis Aktin: Myosin ähnlich 1:7 ist. Dadurch wird die mit höherem Aktin-Gehalt verbundene thixotrope Komponente der Viskosität ausgeschaltet. Gereinigte Aktine geben bei diesem Mischungsverhältnis eine ATP-Empfindlichkeit von 120% (0.6 *M* KCl, 0.001 *M* $MgCl_2$, 0.001 *M* Histidin-Puffer).

Die Spaltungsaktivierung der L-Myosin-ATPase erfolgt in Aktin–L-Myosin-Mischungen von etwa 1:9 und ähnlich 1:4 (0.003 *M* ATP, 0.003 *M* $MgCl_2$, $\mu = 0.085$).

Zur Bestimmung von gebundenem anorganischem Phosphat, gebundenem Gesamt-Phosphat und gebundenem Adenin werden die Aktin-Lösungen mit kalter 20% Perchlorsäure versetzt (10 Vol. Aktin-Lösung, 1 Vol. Perchlorsäure). Das gefällte Eiweiss wird abzentrifugiert, der Überstand mit KOH neutralisiert. Die Bestimmung der freigesetzten anorganischen Phosphate geschieht sofort, die des ablösbaren Gesamt-Phosphats nach Veraschung mit einem H_2SO_4–$HClO_4$-Gemisch (Konz. H_2SO_4–$HClO_4$ 60%, 3:2 v/v). Die Phosphate werden nach ROCKSTEIN UND HERRON[25], das Adenin spektrofotometrisch gemessen.

Für die Phosphat- und ATP-Bindungsversuche wird 1 mal auf der Ultrazentrifuge gewaschenes Aktin mit ^{32}P bzw. [^{32}P]ATP 3 Stunden bei 0° inkubiert (0.1 *M* KCl, 0.001 *M* $MgCl_2$, 0.001 *M* Histidin-Puffer). Danach wird das F-Aktin aus der Lösung durch erneute Ultrazentrifugation abgetrennt (SPINCO 1.5 Std 100,000 × *g*). Der Ueberstand wird möglichst vollständig vom Pellet abgegossen. Der F-Aktin-Niederschlag wird aus dem Zentrifugenröhrchen herausgekratzt, die gewonnene Menge gewogen und mit konz. H_2SO_4 verascht. Nach der Veraschung wird auf ein bekanntes

Volumen aufgefüllt; an Aliquots erfolgen: Eiweissbestimmungen nach Kjeldahl, P-Messungen und Messungen der Radioaktivität. Die gleichen Messungen werden für den Überstand durchgeführt. Von den Konzentrationen im Niederschlag werden die Konzentrationen des Überstandes subtrahiert. Die im F-Aktin gefundene Radioaktivität dividiert durch die spezifische Aktivität des Überstandes ergibt die angelagerten Phosphatmengen. Zum Nachweis, dass Phosphatmenge: 3 = der aufgenommenen Adenin-Menge (dh. Anlagerung des ganzen ATP-Moleküls) wird das abzentrifugierte und gewogene F-Aktin in wenig Aqua dest. homogenisiert. Dann erhält man eine konzentrierte, pipettierbare Eiweiss-Lösung, da das Aktin z.T. depolymerisiert. Nach Perchlorsäure-Behandlung und Neutralisation folgt für das aufgelöste Aktin-Pellet und den Überstand die Adenin-Messung. Von dem Adenin-Wert für das abzentrifugierte F-Aktin wird ebenfalls ein Abzug für eingeschlossenen Überstand gemacht. Daneben erfolgen auch hier Eiweiss-Phosphat- und Radioaktivitäts-Bestimmungen.

[^{32}P]ATP mit gleichmässiger Signierung aller Phosphate wurde in den Forschungslaboratorien der Zellstoff-Fabrik Waldhof, Mannheim hergestellt. Herrn Professor Dr. REIFF und Herrn Dr. KLEMM danken wir sehr herzlich für die Herstellung von Präparaten mit hoher spezifischer Aktivität. Die Präparate enthalten 70–80 % [^{32}P]ATP, papierchromatographisch zeigt sich daneben nur ADP. Vor Gebrauch werden die Präparate durch Ionen-Austauscher nach dem Verfahren von COHN UND CARTER[27] gereinigt und das [^{32}P]ATP isoliert.

ZUSAMMENFASSUNG

1. Die Untersuchungen über den Nukleosid-Phosphat-Gehalt von G- und F-Aktin sind bisher immer an ungereinigten oder wenig gereinigten Präparaten durchgeführt worden. Es ist nie geprüft worden, ob die Reinigung vollständig war und ob die Reinigungsverfahren nicht zu einer Schädigung der Präparate führen.

2. In F-Aktin Präparaten aus STRAUB'schem Rohextrakt sinkt der Adenin-Gehalt durch wiederholte Waschung auf der Ultrazentrifuge oder $MgCl_2$-Fällung nach BÁRÁNY auf einen konstanten und für beide Verfahren identischen Wert von 16 μMol/g Aktin. Die Konstanz des Adenin-Gehaltes und des Gehaltes an Phosphat ist im allgemeinen nach zweifacher Wiederholung der Reinigung erreicht. Durch beide Verfahren werden ausserdem die Eiweissverunreinigungen des Rohextraktes entfernt.

3. Diese Eiweissverunreinigungen werden dagegen durch mehrmalige isoelektrische Umfällung des unpolymerisierten Rohextraktes nicht entfernt. Die isoelektrische Fällung entfernt nur das verunreinigende freie Phosphat und Nukleosid-Phosphat des Rohextraktes. Das Aktin polymerisiert durch isoelektrische Umfällung spontan.

4. Die ultrazentrifugale Waschung, die $MgCl_2$-Fällung und die isoelektrische Fällung in ATP-Gegenwart schädigen das Aktin nicht. Die Viskosität des F-Aktins, die Aktivierung der ATP-ase von zugesetztem L-Myosin und die ATP-Empfindlichkeit des gebildeten Aktomyosins bleiben bis zu fünfmaliger Wiederholung der Reinigung konstant.

5. Isoelektrische Umfällung in ATP-Abwesenheit ist mit fortschreitendem Verlust von Adenin-Phosphat, ATP-Empfindlichkeit und fortschreitender Abnahme von $Z\eta$ verbunden.

6. Im abgelösten Nukleosid-Phosphat des F-Aktins ist die Proportion von

Adenin zu Phosphat 1:2 wie in ADP. Papierchromatographisch finden sich neben dem ADP ganz schwache Spuren von ATP und AMP.

7. Das G-Aktin und die verunreinigenden Proteine des Rohextraktes enthalten ebenfalls 16 μMol Adenin/g Eiweiss.

8. 16 μMol Adenin/g Eiweiss ergeben für G-Aktin und F-Aktin ein minimales Teilchengewicht des Aktin-Monomers von 62,000.

9. Der Phosphat-Gehalt des gebundenen Nukleosids und die Papierchromatographie zeigen, dass im unpolymerisierten Rohextrakt das Protein-gebundene Nukleosid-Phosphat zu 70–75 % aus ATP, der Rest im wesentlichen aus ADP besteht. An der Polymerisation nehmen nur 70–75 % des Rohproteins teil.

10. G-Aktin, hergestellt aus gereinigtem F-Aktin, enthält ebenfalls nur 70–75 % des Nukleosid-Phosphates als ATP. Es polymerisiert trotzdem praktisch vollständig. Es hängt also von der Vorgeschichte ab, ob ADP-G-Aktin polymerisiert oder nicht.

11. G-Aktin, dessen Polymerisationsfähigkeit durch Röntgenbestrahlung weitgehend zerstört ist, aktiviert die L-Myosin-ATP-ase immer noch in völlig normalem Umfang. Das gleiche gilt für Aktin, das durch isoelektrische Umfällung in ATP-Abwesenheit denaturiert ist. Polymerisationsfähigkeit und Fähigkeit der Aktivierung der L-Myosin-ATP-ase sind also voneinander unabhängig.

12. Gereinigtes F-Aktin bindet in Phosphat- oder ATP-Lösungen ATP und Phosphat reversibel (zusätzlich zu dem festgebundenen ADP). Dagegen findet auch in ATP-Anwesenheit eine Phosphatbindung durch Transphosphorylierung nicht statt.

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THE ACTIVE TRANSPORT OF SUGARS BY VARIOUS PREPARATIONS OF HAMSTER INTESTINE*

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SUMMARY

The active transport of sugars by the small intestine of the golden hamster has been studied *in vitro* by means of the accumulation of sugar within the tissue against a concentration gradient. Techniques are described for preparing and using three types of intestinal preparation which exhibit characteristics of active transport similar to those of other *in vitro* preparations and of active absorption *in vivo*. One kind, in particular, which consists of small rings of everted intestine was shown to be highly reproducible and, therefore, useful for comparison of the rate and extent of active transport under various conditions. The influence of fasting on active sugar transport was studied and the results are discussed with respect to the source of energy for active transport. The variation in the rate of active transport along the length of the intestine was studied with glucose, galactose and 1,5-anhydro-D-glucitol and found to be similar for all three compounds.

INTRODUCTION

The fact is experimentally well-established that the small intestine possesses an energy-dependent transport process by means of which glucose and certain other sugars can be absorbed against an apparent concentration gradient. This phenomenon has been demonstrated, *in vivo*[1-3], during absorption of sugar from the lumen of intestinal segments into the blood stream and, *in vitro*[4-8], during transfer of sugar from the mucosal to the serosal surface of preparations of intestinal tissue. Since it has also been repeatedly shown that the absorbed or transferred sugar is in a "free" state, there is little doubt that the phenomenon is one of "active transport" as defined by ROSENBERG[9]. However, many characteristics and the mechanism of the process remain largely unknown.

The *in vitro* techniques cited above[4-7] have made it possible to study some properties of the process of active transport such as its response to the cationic environment of the medium[10,11], its specificity with respect to the configuration of

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Abbreviations: PPO, diphenyloxazole; POPOP, bis-phenyl oxazolylbenzene.

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sugars presented for absorption[12–15], and its rate with respect to their concentration[7,10,16,17]. However, the significance of much of the collected data suffers from an inherent disadvantage of these techniques. In all of them, the intestine is mounted or prepared in such a way that the medium is separated into two compartments, one bathing the mucosal surface and the other bathing the serosal aspect of the intestinal wall and sugar which is actively transported by the epithelial cells must traverse the entire intestinal wall in order to be measured. Thus, the observed properties of the active transport system include the characteristics not only of the uptake of sugar from the mucosal compartment into the epithelial cells[18] but also of its diffusion through several cell layers as it passes into the serosal compartment of the preparation. Since there is reason to believe that the serosal portion of the intestinal wall is a quantitatively important barrier to diffusion[19], it may be surmised that parameters measured by these methods will have different values than if measured under conditions where this unphysiological diffusion barrier does not exist.

In order to overcome this objection and yet retain the utility of an *in vitro* method, studies have been carried out in which active transport has been measured by means of the accumulation of sugar against an apparent concentration gradient within the tissue itself. With such a method, sugar need enter only the epithelial cells in order to be measured and the underlying tissues serve in these preparations as a repository of transported sugar analogously to the serosal medium in the other *in vitro* methods. In some respects, the experiments are similar to those of KRANE AND CRANE[20] in which it was possible to observe an accumulation of D-galactose against an apparent concentration gradient by slices of rabbit kidney cortex. Previous studies by AGAR, HIRD AND SIDHU[21,22] have shown that surviving strips of intestine can accumulate L-amino acids against an apparent concentration gradient.

MATERIALS AND METHODS

Golden hamsters of either sex and weighing from 150 to 250 g were killed by stunning. The small intestine was removed, flushed with 0.9 % sodium chloride solution, turned inside out[6] and washed in KREBS–HENSELEIT bicarbonate buffer[23].

Tissue preparations

From the washed, everted intestine, four different kinds of preparations were then made as desired. Intact strips: The entire everted intestine was cut with scissors into ring-like pieces having a breadth of 2 to 4 mm. Regions rich in lymphoid tissue (Peyer's patches) were discarded. The pieces were taken for individual flasks in rotation to approximate random sampling and were kept in buffer at room temperature (about 25°) until used. The bicarbonate buffers were maintained in an atmosphere or under a jet of O_2–CO_2 (95:5) at all times, including the time during transfer of tissue or the addition of reagents.

Mucosal and serosal sheets. By means of the technique described by DICKENS AND WEIL-MALHERBE[24], larger segments of intestine were cut apart at about the level of the *muscularis mucosae* into two morphologically distinct sheets of tissue, namely, "mucosal sheets" which were composed, for the most part, of villi held together by the underlying *muscularis mucosae* (see Plate 1) and "serosal sheets" which were

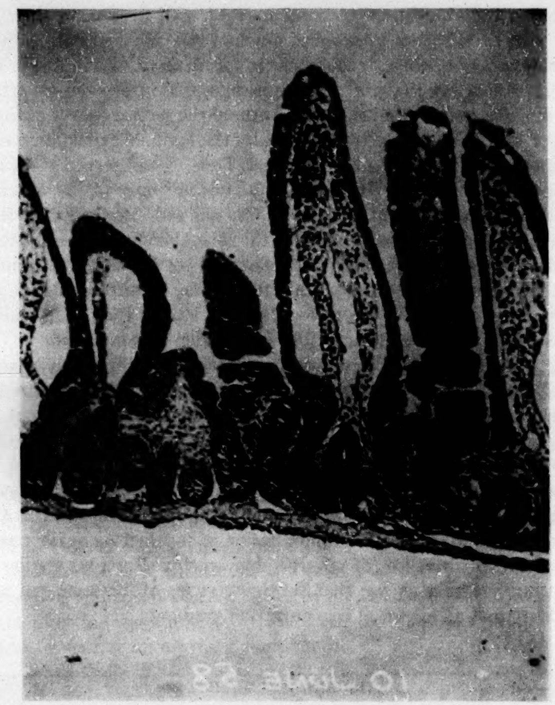

Plate 1. Section of mucosal sheet, fixed in Bouin's fluid and stained with haematoxylin and eosin. Photograph taken under phase contrast illumination, 100 ×.

composed of the submucosal layer, two layers of muscle, the serosa and a few isolated patches of villi (see Plate 2).

Villi: Preparations having no intact structure other than the villi themselves were obtained by fragmentation of mucosal sheets. The sheets were transferred to a 20-ml syringe together with about 10 ml of bicarbonate buffer and forced through needles of successively smaller size. A number 15 needle was used first and was followed by a number 18, then a number 19, and, occasionally when necessary for more complete fragmentation, a number 21. After each treatment, the fragmented tissue was collected by pouring the suspension onto number 16 bolting silk. The tissue retained by the silk was collected by scraping with a spatula and resuspended in buffer. Because the mucous secretions of the intestine unduly lengthened the time required for filtration, the mucosal sheets were incubated for 5 min at 30° in 5 ml of buffer containing 5 mg of a hyaluronidase preparation prior to the fragmentation steps. A bovine testicular hyaluronidase preparation having an activity of 1380 USP units/mg which was a gift of the Wyeth Institute for Medical Research was routinely used, but a less active preparation obtained from Worthington Biochemical Corporation gave similar results

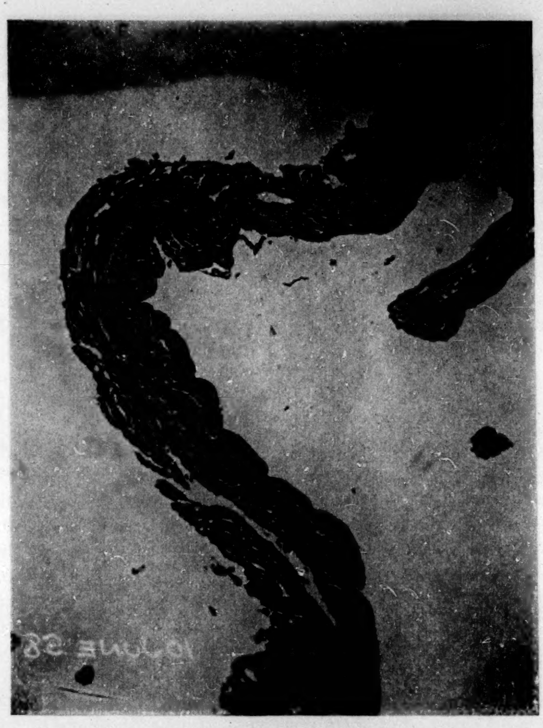

Plate 2. Section of serosal sheet, fixed in Bouin's fluid and stained with haematoxylin and eosin. Photograph taken under phase contrast illumination, 100 ×.

when used in twice the amount. Comparative experiments showed that pretreatment with hyaluronidase never lowered the observed transport activity of the villi preparations and frequently increased it. These preparations of villi, each of which was examined by phase contrast microscopy before use, contained numerous single villi (Plate 3) together with clumps in which from 2 to 6 villi were joined together by one edge of their bases.

Techniques for incubation

Intact strips, mucosal sheets and serosal sheets were incubated in rubberstoppered Erlenmeyer flasks in a shaking incubator of the Dubnoff type manufactured by the Precision Scientific Company. Flasks containing the desired volume of bicarbonate buffer were brought to temperature equilibrium and the tissue was introduced. After an additional 2–3 min period of temperature equilibration, an aliquot of the desired sugar solution was added and incubation was continued for the desired period of time. Incubation was terminated by the removal of the tissue. Except for the mucosal sheets which because of their fragility were weighed before they were introduced into the

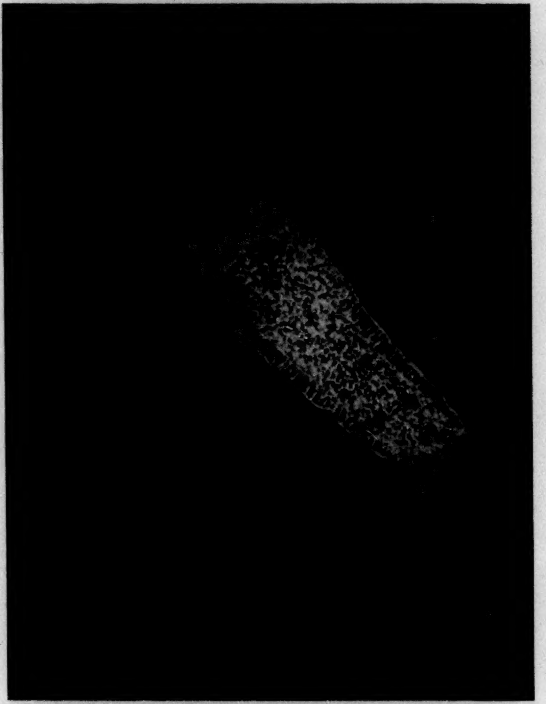

Plate 3. **Intact villus. Photograph taken under phase contrast illumination, 100 ×. The dark areas are stained with nigrosin which was added as a test of membrane integrity[54].**

flasks for incubation, the recovered tissues were blotted gently on filter paper and weighed on a torsion balance. They were then transferred to 2 ml of 0.19 *M* zinc sulfate solution contained in a conical glass homogenizing tube fitted with a motor-driven ground conical pestle. After homogenization, 2 ml of 0.3 *N* barium hydroxide solution were added and mixed in. The tubes were then centrifuged. In some experiments 4 ml of trichloracetic acid (5 g/100 ml) were used to deproteinize instead of zinc sulfate and barium hydroxide. The protein-free solutions were diluted appropriately with water to bring the concentration of sugar within the range of the applicable method of assay.

Villi preparations were incubated in specially designed glass tubes having a volume of about 8 ml. These tubes were about 12 cm in overall length and were constructed by sealing pyrex tubing of 9 mm, ID, to tubing of 1.5 mm, ID. The tube of narrower diameter was about 2.5 cm in length and was closed at one end with a flattened seal so that it could serve as a cytocrit tube. The volume of a given length of this narrower tube was determined by calibration with mercury. The length of the wider tube was adjusted to fit the International Clinical Centrifuge and the open end was flared slightly to admit a size O rubber stopper. Aliquots, usually 2 ml, of a suspension of villi were added to the tubes by pipet. The tubes were stoppered and incubated in a vertical position without shaking for 3 min to achieve temperature equilibration. They were then opened to introduce the sugar solution, resealed and incubated in a horizontal position with gentle shaking for the desired period of time. Care was taken throughout to handle the tubes so that the medium and its suspended villi did not enter the capillary portion of the tube prior to centrifugation. Incubation was terminated by centrifugation for 5 min at about 900 × *g*. The length of the sedimented column of villi was measured, the supernatant fluid was decanted, and the tube, including the portion of the capillary above the packed villi, was dried with absorbent tissue and pipe cleaners. The tube was washed with wetted paper and cleaners and again dried. Then 1.5 ml of zinc sulfate solution were added and the packed villi were suspended by means of a syringe and needle. An equal volume of barium hydroxide solution was then added and mixed in by vigorous strokes of the plunger. Alternatively, in some experiments, 1 to 3 ml of 5 g/100 ml trichloracetic acid were used. Further steps in the preparation for analysis were substantially the same as those described above.

Methods of assay

Glucose was assayed spectrophotometrically by means of the glucose oxidase reaction[25,26] using the glucostat reagent supplied by the Worthington Biochemical Corporation or with the coupled hexokinase, glucose-6-phosphate dehydrogenase reaction[27] using enzymes and triphosphopyridine nucleotide purchased from the Sigma Chemical Company. Galactose was assayed colorimetrically by the SOMOGYI method[28] or, when [1-^{14}C]galactose was used, by virtue of its radioactivity. Non-radioactive 6-deoxy-D-glucose was assayed by the DISCHE AND SHETTLES procedure[29] which is specific for methyl pentoses. The modified ROE method[30] was used for assay of pentoses and the colorimetric procedure described by MCPHERSON[31] was used for histidine.

6-deoxy-D-glucose, 1,5-anhydro-D-glucitol, 1,5-anhydro-D-mannitol, 2-deoxy-D-glucose, and 3-O-methyl-D-glucose were each made radioactive by exposure in the dry state to tritium gas[32] and purified by chromatographic procedures[33]. These compounds, as well as [1-^{14}C]galactose, were assayed by a liquid scintillation technique with the Packard Tri-Carb liquid scintillation spectrometer. To aliquots of deproteinized solutions dried directly in the counting vials, 2 ml of absolute ethanol were added. The vials were then closed and shaken mechanically for 30 min or allowed to stand overnight with occasional shaking by hand. Then 10 ml of toluene containing PRO, 4 g/l, and POPOP, 0.1 g/l, were added and mixed in by swirling. Tests of this procedure in which graded quantities of the non-radioactive species were added together with the tritium-labeled compounds indicated quantitative elution of sugar from the dried residue and satisfactory solubility of the eluted compounds in the scintillation mixture. The observed specific activity of the tritium labeled compounds ranged from 5 to 60 million counts/min/mg. In all experiments, an amount of each radioactive compound was used which would provide for analysis a counting rate at least 10 times background. The non-radioactive analogue was added in the amount necessary to produce the desired final concentration.

Calculation of data

Data are reported as millimolar concentrations of sugar in the tissue and in the medium or as the ratio of these concentrations, T/M. In order to do this, certain assumptions had to be made in the calculation of tissue concentrations. It was assumed that the sugar was uniformly distributed throughout the tissue water. It would have been preferable to have analysed each tissue layer separately to locate the actively transported sugar as has been done with galactose by McDougal, Little and Crane[18]. However, methods of the required sensitivity are not yet available for most sugars. The assumption made, seems to be the one least prejudiced in favor of high tissue to medium concentration ratios. The value of tissue water for intact strips, mucosal sheets and serosal sheets was assumed to be a constant fraction of the wet weight of the blotted tissue. The value was experimentally determined only for intact strips by finding the difference in weight of representative strips before and after drying to constant weight at 100° *in vacuo*. The average value found in the samples used was 82.5 % of the wet weight; the value used in the calculations was 80 %. A correction for the amount of sugar which entered the tissue by routes other than through the active transport process was also made in some experiments. This correction was based on the tissue concentration after 15 min of incubation at 37° of a compound, 1,5-anhydro-D-mannitol, which is not actively transported[12]. This concentration was found to be close to 30 % of the medium concentration. In the strict sense, this correction, although similar in value to extracellular space corrections for other tissues[20, 34], is not a correction for extracellular space but for passive diffusion of sugar into the tissue. Calculations of tissue concentrations in intact strips, thus, took the following form:

$$\text{m}M\ (\text{tissue}) = \frac{\text{m}M\ (\text{filtrate}) \times \text{homogenate volume}}{\text{wet weight of tissue} \times 0.8} - 0.3 \times \text{m}M\ (\text{medium})$$

Since the correction for passive diffusion into mucosal sheets and serosal sheets was not experimentally determined the correction, 0.3 × mM (medium), was omitted in the calculations for these preparations.

Calculations of tissue concentrations for villi take a slightly different form because of the technical differences in the experiments. Recent measurements of the apparent extracellular volume of the packed villi have been made by Dr. I. Bihler, using [1-^{14}C]mannitol. This compound can be used for this purpose with erythrocytes[34] and the isolated perfused heart[35] and has been shown to be not absorbed from an intestinal loop in the intact rat[37]. The measurements with villi indicate a value in the range of 75 to 80 % for the extracellular volume. A volume of 75 % has been assumed. Since the villi are measured by volume rather than weight, no correction for the space occupied by tissue solids has been made. Calculation of tissue concentrations in villi, thus, took the following form:

$$\text{m}M\ (\text{tissue}) = \frac{\text{m}M\ (\text{filtrate}) \times \text{homogenate volume} - 0.75 \times \text{villi volume} \times \text{m}M\ (\text{medium})}{0.25 \times \text{villi volume}}$$

EXPERIMENTAL

Studies with intact strips, general

Active transport of sugar by intact strips of intestine was found, like active transport of sugar by other *in vitro* preparations[4–6], to be characterized by (a) dependence on aerobic metabolism, (b) inhibition by phlorizin and (c) specificity with respect to the sugars that are actively transported. For example, as shown in Table I, 6-deoxy-D-glucose was accumulated within the tissue to a high concentration relative to the medium, T/M = 17.2. Accumulation did not occur when the tissue was deprived of oxygen, when 4,6-dinitro-*o*-cresol, a potent inhibitor of aerobic phosphorylation[38], was added nor in the presence of phlorizin. Moreover, the specificity of active transport by this preparation, insofar as it was examined (see Table II), paralleled the findings with the everted sac preparation[12–15]. All actively transported compounds have, in common, a pyranose ring, a methyl or substituted methyl group at carbon-5 of the ring and a hydroxyl group in the glucose configuration at carbon-2. Compounds which lack one or more of these structural features are not actively transported.

TABLE I

ACTIVE TRANSPORT OF SUGARS BY INTACT STRIPS OF HAMSTER INTESTINE UNDER VARIOUS CONDITIONS

Incubations were at 37° for 40 min. Concentrations of sugars and added components were as follows: 6-deoxy-D-glucose, 0.25 m*M*, 3-O-methyl-D-glucose, 1.0 m*M*, D-xylose, 1.0 m*M*, phlorizin, 1.0 m*M*, and 4,6-dinitro-*o*-cresol, 0.1 m*M*. Nitrogen refers to a gas space of N_2–CO_2 (95:5) instead of the usual oxygen–carbon dioxide mixture. Animals were fasted 16 h. The data for D-xylose are not corrected by the factor of 0.3 × m*M* (medium). T/M is the ratio of the final tissue concentration to the final medium concentration.

Test sugar	*Condition*	*T/M*
6-deoxy-D-glucose	Control	17.2
	Nitrogen	0.7
	Phlorizin	0.7
	4,6-dinitro-*o*-cresol	1.0
3-O-methyl-D-glucose	Control	3.4
	Nitrogen	0.6
D-xylose	Control	0.4
	Nitrogen	0.3

TABLE II

ACTIVE TRANSPORT OF VARIOUS SUGARS AND RELATED COMPOUNDS BY INTACT STRIPS OF HAMSTER INTESTINE

Incubations were at 37° for the time periods indicated. T/M is the ratio of the final tissue concentration to the final medium concentration.

Compound	*Incubation period (min)*	*Concentrations, mM* *Medium*		*Tissue*	*T/M*
		Initial	*Final*		
Glucose	20	1.0	0.07	14.2	203
	30	4.0	1.46	32.0	21.8
Galactose	20	4.0	1.81	33.0	18.2
3-O-methyl-D-glucose	40	3.8	3.3	7.6	2.3
1,5-anhydro-D-glucitol	40	1.0	0.94	5.5	5.8
6-deoxy-D-glucose	35	0.25	0.084	2.12	25.3
7-deoxy-D-glucoheptose	30	1.0	0.73	12.6	17.3
	30	0.073	0.013	1.06	82
2-deoxy-D-glucose	15	1.2	1.2	0.37	0.31
1,5-anhydro-D-mannitol	15	1.2	1.2	0.30	0.25
D-xylose	40	5.0	4.9	1.5	0.38
L-arabinose	30	1.0	1.0	0.30	0.30

The final relative concentrations between tissue and medium, T/M, shown in Table II are of additional interest in that they provide insight into the concentrative potential of the active transport process. At low external concentrations, glucose, for example, was accumulated to a T/M of over 200 and 7-deoxy-D-glucoheptose to T/M of over 80. In view of the fact that these values are based on average tissue concentrations, it is very likely that the T/M between the epithelial cells and the medium is even higher[18]. Under these conditions, only a small absolute amount of sugar was accumulated. When higher concentrations were used, the value of T/M was lower. The apparent concentration gradients for glucose are especially noteworthy because accumulation of this sugar reflects the extent to which active transport is more rapid than utilization. The differences in observed T/M among the sugars are large and it is likely that one reason for these differences is the differences in the apparent K_m values for the transport process[39]. When sugars with different K_m values are present at the same initial non-saturating concentration, differences in the rate of transport and, consequently, in the extent of accumulation may be expected.

The final relative concentrations of compounds which were not actively transported was in the range 0.3 to 0.4. It would be reasonable to expect that T/M for these compounds would approach or equal 1. Why it did not do so is not clear from the presently available information. One would suppose that sugars could gain entrance to the total extracellular space of the tissue and thus to all of the cells by means of the cut margins of the strips. To explain the apparent restriction of these sugars to a tissue volume which is not much, if any, larger than the extracellular space one may suppose either (a) that the cells of the tissue (muscle cells, epithelial cells, submucosa, etc.) are relatively impermeable to these sugars or (b) that these sugars enter the cells at a rate which is not much greater than the rate at which they are utilized. Recent studies indicate that a somewhat greater volume of distribution can be obtained if incubation is prolonged for a period of 1 h or more.

Reproducibility

A major advantage of the technique with intact strips is its utility for experiments containing multiple variables. With the usual type of *in vitro* preparation in which a long segment of the intestine is used, precise kinetic studies on a routine basis have not been possible owing to variations in the rate of active transport throughout the length of the small intestine[40]; even adjacent segments exhibit significantly different rates. To get around this difficulty, resort has been made to reusing the same segment of intestine during several successive time periods[7] or to using the entire intestine from a large series of animals[5, 16, 17]. However, kinetic studies are most easily performed if each of several flasks in a single experiment contains tissue of the same or nearly the same potential activity. The present technique, as the results in Table III indicate, provides the required degree of reproducibility to make this possible. As is described in the legend to this table, intact strips from the intestine of one animal were divided among three flasks and incubated with sugar under identical conditions. The amount of accumulated tissue sugar, either glucose or galactose, was then measured. It was found to vary within reasonably small limits. Since both glucose and galactose are utilized by the intestine, the use of these two sugars is believed to represent the most stringent test of the technique.

TABLE III

STUDIES ON THE REPRODUCIBILITY OF THE MEASUREMENT OF ACTIVE TRANSPORT OF SUGARS WITH INTACT STRIPS OF HAMSTER INTESTINE

Each experiment was carried out with the intestine from a single animal divided among three flasks as described in the METHODS section. The temperature of incubation was 37°. The initial concentration of sugar was 5 mM in all cases.

Sugar	*Duration of fast (h)*	*Duration of incubation (min)*	*Concentration mM*	
			Final medium	*Tissue*
Glucose	48	10	2.47 ± 0.35	30.0 ± 1.2
	48	15	2.06 ± 0.33	34.8 ± 2.1
Galactose	24	10	2.43 ± 0.19	26.7 ± 1.9
	24	20	2.05 ± 0.25	32.4 ± 2.9
	48	15	2.76 ± 0.09	27.1 ± 0.51
	72	15	2.72 ± 0.24	22.3 ± 0.54
	96	15	2.40 ± 0.10	39.3 ± 1.2

The effect of fasting

Since tissue from non-fasted animals was found to produce significant quantities of free glucose during incubation (for example, 350 mg of tissue from a non-fasted animal produced, in 5 min of incubation, 1 mM of glucose in the tissue water and 0.03 mM in the 4 ml of medium) and tissue from fasted animals yielded no detectable free glucose, experiments were carried out to see whether fasting would be deleterious to the active transport process. Earlier studies[41, 42] in the intact animal indicated that fasting for periods of time similar to those used in the present study greatly reduced intestinal absorption of sugar. Careful studies of the effect of fasting periods of less than 24 h have not been done, so it is possible that during the first day there is a loss in the transport activity of the intestine as measured by the intact strip technique. However, from the 24th to the 96th h of fasting, (see Table III) there was no continued loss with time and there was not more difference than may have been expected as a variation between individual animals. In fact, the greatest accumulation was observed in strips from the animal that had been fasted for 96 h. Some of the experiments in the present report were carried out prior to the studies on the effect of fasting and, as noted in the legends, the animals were fasted for only 16 h. For all other experiments in this and subsequent reports the animals were fasted at least 48 h prior to use.

Location along the intestine of the active transport process

FISHER AND PARSONS[16, 17, 43], using a different *in vitro* technique[4] and a different species, found different relative rates of active transport for glucose and galactose along the length of the intestine and suggested on that basis that the two sugars may be absorbed by separate processes. On this account, the pattern of variation in the extent of active transport measured by the intact strip technique was studied. For these experiments, the intestine from a single animal was used for each sugar studied. The excised and everted intestine was laid, without stretching, alongside a 30 cm rule and successive 4–5 cm portions, beginning at the pylorus, were taken and separately cut into the usual small pieces. The pieces were incubated in vessels identified as to the portion of the intestine from which they were obtained and were kept in order throughout the rest of the procedure. Three sugars were used; namely, glucose, galactose and the non-utilizable compound 1,5-anhydro-D-glucitol. The results of these experiments are shown in Fig. 1. In all three experiments, the greatest extent of accumulation was found with tissue taken from the middle portion of intestine.

Rate and extent of accumulation

Accumulation of sugar with the formation of an apparent concentration gradient between the tissue and the medium occurred rapidly at 37°, as is illustrated by the time courses of accumulation of the sugars, glucose and galactose, shown in Fig. 2. The initial rate, however, was not maintained; it fell off with time as an apparent maximal extent of accumulation was attained. Although the maximal value was not measured in the experiments shown in Fig. 2, it was usually found in other experiments to be about 30–40 m*M* and to be attained in the time period between 15 and 20 min.

Calculations of initial rates from the curves presented in Fig. 2 and comparison of these rates with some values for various metabolic parameters collected from the literature (Table IV) show active sugar transport to be a major activity of intestinal tissue. Glucose transport measured by the strip technique is more than three times faster, on a molar basis, than oxygen consumption, lactate formation or glucose utilization. Galactose transport is 16 times more rapid than galactose utilization. The strip technique shows, moreover, a 2–3 fold higher rate of transport than the reported value for everted sacs. The value of 1080 μmoles/g wet weight/h for galactose transport is taken from a later paper of this series[18] in which analyses of the epithelial cell layer

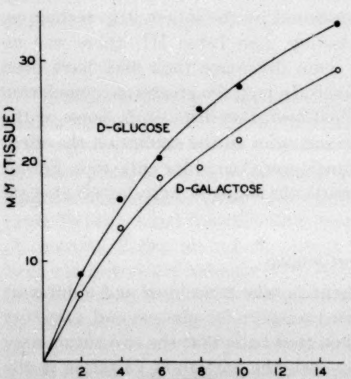

Fig. 1. The distribution of active sugar transport activity along the length of the hamster intestine. The temperature of incubation was 37°. The initial concentrations were, as follows: galactose, 10 m*M*, glucose, 5 m*M*, and 1,5-anhydro-D-glucitol, 5 m*M*. The duration of all incubations was 15 min.

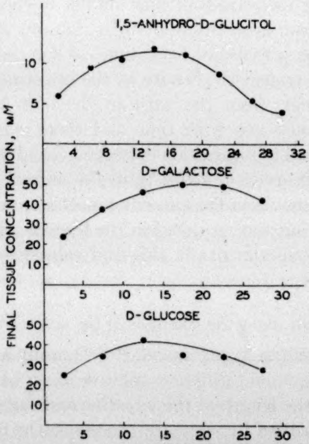

Fig. 2. The accumulation of sugar within intact strips as a function of time. Temperature of incubation was 37°. The initial concentration of sugar was 5 m*M* in both instances. The rates are not be to compared as intestine from different animals was used for the two sugars.

TABLE IV

RATES OF VARIOUS METABOLIC ACTIVITIES AND OF ACTIVE SUGAR TRANSPORT FOR HAMSTER INTESTINE

Values taken from the work of others are average values of the published data and they have been corrected to a wet weight basis assuming a dry to wet weight ratio of 0.20. Except for the single value marked with an asterisk, which is based on the wet weight of the epithelial cell layer, all weights refer to the entire intestinal wall.

Activity measured	*µmoles/g wet weight/h*	*Reference*
Active transport by the strip technique:		
Glucose active transport	417	See text
Galactose active transport	338	See text
	1080*	See text
Active transport by the everted sac technique:		
Glucose active transport	152	44
Galactose active transport	134	44
Oxygen consumption	123	45
Aerobic lactate formation	59	45
Anaerobic lactate formation	132	45
Aerobic glucose utilization	124	6
Aerobic galactose utilization	20	46

were performed. If this rate were calculated on the basis of the wet weight of the entire intestinal wall, it would be somewhat greater than the other values obtained with the strip technique.

The results of studies of the influence of the initial concentration of sugar in the medium on the apparent final tissue concentration and the final relative concentrations, T/M, are shown in the diagrams in Fig. 3. Both parameters were influenced by

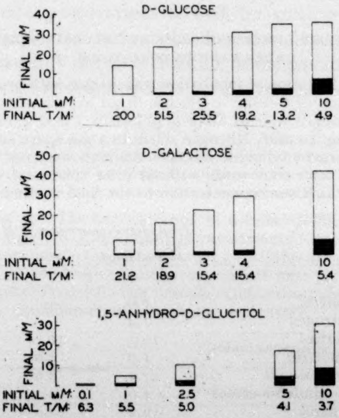

Fig. 3. The active transport of sugars by intact strips as a function of concentration. The total height of the column represents the tissue concentration; the height of the cross-hatched portion, the final concentration in the medium. Incubation temperature was 37°. Duration of incubation was 20 min for glucose and galactose, 15 min for 1,5-anhydro-D-glucitol.

the initial concentration of sugar. In part, this must have been due to the influence of the concentration on the rate of transport inasmuch as the range of concentrations used included the apparent K_m (see ref. 39). However, comparison of the data for all three sugars at the 10 mM concentration level suggests the possibility that there is a limiting concentration which the tissue can accumulate. This aspect will be the subject of future investigations, since studies in this area should provide some insight as to whether the observed concentrations should be viewed as the result of a unidirectional inward active transport of sugar balanced at a steady-state by outward diffusion, or in some other way.

Studies with mucosal sheets, serosal sheets and villi

Mucosal sheets and villi are able to accumulate sugar against an apparent concentration gradient and the accumulation is inhibited by anaerobiosis, or the addition of phlorizin, 4,6-dinitro-*o*-cresol, or a second actively transported sugar (Table V). In these ways, active transport in mucosal sheets and villi is like active transport in intact strips. The mucosal sheets, owing to their fragility, are not well suited to the routine study of active transport, but the villi seem to be. *In vivo*, the villus is the physiological absorption unit; that is, sugar transported by the epithelial cells passes into the "core" of the villus where it enters the blood stream. The villi preparation is a means for studying some of the characteristics of this physiological unit. The initial rate of accumulation using 6-deoxy-D-glucose as the substrate varied widely with villi preparations from different animals but it was usually in the range of 20–80 µmoles/ml intracellular water/h. Oxygen consumption of villi prepared from the intestine of a 48-h fasted animal and incubated without added substrate was about 280 µmoles/g wet weight/h; a value which is comparable to the oxygen consumption value given in Table IV for everted sacs. With villi, as with intact strips, the rate of

TABLE V

ACTIVE TRANSPORT OF 6-DEOXY-D-GLUCOSE BY "MUCOSAL SHEETS" AND VILLI UNDER VARIOUS CONDITIONS

Incubation temperature 37°. Duration of incubation was 40 min with mucosal sheets, and 15 min with villi. Initial concentration of 6-deoxy-D-glucose in all experiments was 0.25 mM. Concentrations of other added compounds were as follows: phlorizin, 1 mM 4,6-dinitro-*o*-cresol, 0.1 mM, galactose, 10 mM, and glucose, 10 mM. Nitrogen refers to a gas space containing N_2–CO_2 (95:5) in place of the usual oxygen–carbon dioxide mixture. Animals were fasted 16 h. The villi for the experiments reported in this table were made without prior treatment with hyaluronidase. T/M is the ratio of the final tissue concentration to the final medium concentration.

	Final relative concentration, T/M	
	Mucosal sheets	*Villi*
Control	6.6	—
+ Nitrogen	1.4	—
Control	12.5	10.2
+ Phlorizin	1.9	1.0
Control	27.2	8.6
+ 4,6-dinitro-*o*-cresol	2.0	2.2
Control	16.2	23.0
+ Galactose	5.6	7.8
Control	11.1	27.0
+ Glucose	2.4	1.4

sugar accumulation fell off rapidly with time and the extent of accumulation was maximal in about 10–15 min. Although one might have expected the villi to be a very "leaky" preparation, inasmuch as sugar which leaves the serosal side of the epithelial cell is returned to the medium rather than trapped in the underlying tissues as it is in the intact strip, the gradients which are established (Table V) are large. This conforms with the finding of McDougal *et al.*[18] that sugar is accumulated within the epithelial cells.

It was found instructive to compare active transport in the intact strip with active transport in its sub-units, the mucosal sheet and serosal sheet. The results of these studies are shown in Table VI. On the one hand, the intact strip was incubated in a medium containing 6-deoxy-D-glucose. After incubation, it was separated as described in the section on MATERIALS AND METHODS into a mucosal sheet and a serosal sheet and the separated portions were analyzed separately. On the other hand, the separation was made prior to incubation and the mucosal sheet and serosal sheet were incubated separately. When separation was made after incubation it was found that both portions contained accumulated sugar and at approximately equal concentrations. However, when separation was made before incubation, only the mucosal sheet contained sugar at a high relative concentration. Obviously, the epithelial cells are required for active transport of sugar. This difference between the preparations, however, does not extend to the transport of amino acids. The data in Table VI show that the amino acid, L-histidine, is accumulated by both mucosal and serosal sheets when incubated separately. It thus appears amino acid can be accumulated within the tissue independently of the epithelial cells.

DISCUSSION

Data presented above demonstrate that accumulation of sugar to high relative tissue concentrations with *in vitro* preparations of hamster small intestine can serve as a highly reproducible system for the study of the characteristics of intestinal active sugar transport. With the availability of this technique, it was possible to make a number of other observations which are relevant to certain aspects of the active transport process.

TABLE VI

THE ROLE OF THE MUCOSAL AND SEROSAL PORTIONS OF THE INTESTINAL WALL IN THE ACCUMULATION OF 6-DEOXY-D-GLUCOSE AND L-HISTIDINE BY INTACT STRIPS OF HAMSTER INTESTINE

Incubation temperature was 37°. The concentration of 6-deoxy-D-glucose was 0.25 m*M* and of L-histidine 2.48 mg/ml. The duration of incubation in experiments A and C was 30 min, in Expt. B, 22 min. For further details consult the text. The data for L-histidine are not corrected for the factor 0.3 m*M* (medium). In Expt. A the mucosal and serosal sheets were prepared from an intact strip after its incubation. In Expts. B and C, the mucosal and serosal sheets were prepared before incubation and incubated separately.

Expt.	*Compound*	*Relative concentration, T/M*		
		Intact strip	*Mucosal sheet*	*Serosal sheet*
A	6-deoxy-D-glucose	28.6	28.2	30.6
B	6-deoxy-D-glucose	—	15.1	1.4
C	L-histidine	4.8	3.8	2.9

First, there is the question of the source of the energy which is required for accumulation. When glucose and galactose serve as the substrate for active transport they are also utilized and energy generated by their utilization may provide the necessary driving force. However, when non-metabolizable compounds are accumulated, energy is probably derived from another source. In the experiments reported in Fig. 3, the animals had all been fasted for 48 h. No utilizable substrate was added, yet nearly as much 1,5-anhydro-D-glucitol as glucose or galactose was accumulated in an equivalent time period. Oxygen consumption of intact strips and of villi was rapid and did not depend on the addition of substrate. In fact, several unsuccessful attempts were made to influence appreciably the endogenous oxygen consumption of villi by the addition of various sugars, Krebs cycle intermediates, etc. These observations suggest that energy for the active transport of sugars under the present experimental conditions is provided by the oxidation of non-carbohydrate metabolites, possibly fat or fatty acids. It would seem relevant to this point that DICKENS AND WEIL-MALHERBE[24] observed with rat intestinal mucosa a low respiratory quotient which was not greatly influenced by the addition of glucose to the medium.

Second, there is the question of the location of the active transport process in different regions of the small intestine and in other parts of the gastrointestinal tract. Tests of active sugar transport with stomach and proximal colon of the hamster were made by the intact strip technique and compared to the results obtained with intact strips of small intestine prepared from the same animal. Following incubation at 37° in a medium containing 6-deoxy-D-glucose the small intestine was found to have accumulated this compound to a final relative concentration of $T/M = 25.4$, whereas for the stomach the final relative concentration was $T/M = 0.62$ and for large intestine, $T/M = 0.61$. The active process, then, is restricted to the small intestine, though not to as limited a portion of it as appears, for example, to be true for active Ca^{++} transport[47]. The activity is present throughout the small intestine although it varies along its length with the greatest extent of accumulation measured by the intact strip technique observed in the mid-portion (Fig. 1). In previous studies with a different technique[4] in the rat, FISHER AND PARSONS[48] observed a progressive diminution in the rate at which glucose moved from the mucosal into the serosal compartment with the distance of the segment used from the pylorus. With galactose, however, the middle third of the intestine was found to be most active[17]. As mentioned above, this apparent difference in localization led FISHER AND PARSONS to suggest that glucose and galactose are transported by different mechanisms or different cells. However, since the observations made by FISHER AND PARSONS with glucose diverge not only from their own results with galactose but also from those obtained in the present studies with glucose, galactose and 1,5-anhydro-D-glucitol, it would seem possible that the explanation for the divergence may be found in the characteristics of glucose transport *in vitro* rather than in the location of the active transport process. For instance, in the measurement of the glucose transferred in the *in vitro* preparation used by FISHER AND PARSONS[16] an appreciable quantity of endogenous glucose was formed during the experiment and this endogenous glucose contributed to the measured rate of glucose transport. Endogenous stores of glucose (glycogen) may be expected to be greater in the cells more proximal to the pylorus inasmuch as these are exposed during digestion to high luminal concentrations of glucose[19]. Also, utilization of glucose accounted for an appreciable fraction of the glucose which disappeared from the mucosal compartment in the experiments of FISHER AND PARSONS. It was assumed by these authors in their interpretation of the results that glucose which is transported enters the mucosal cells by a different portal than glucose which is metabolized.

If, on the contrary, it is assumed that all glucose enters the epithelial cells by the same portal and that the total glucose disappearance is equal to the rate of transport, the gradient calculated from the data of FISHER AND PARSONS[48] would be much less steep than the one presented. Experiments reported in a later paper of this series[18] show that transported sugar is accumulated within the epithelial cells and it seems reasonable to conclude that the same glucose pool contributes to diffusion into the submucosal tissues and to utilization. In experiments with galactose, the possible contributions of endogenous sugar and utilization to the measured rate of transport are greatly reduced, since there are no large tissue stores of galactose and galactose is not well-metabolized by the intestinal mucosa. It may be expected that the rate at which galactose is transferred will be more nearly representative of the rate of active transport by the epithelial cells.

Third, there is the question of the identification of the process by which sugar is accumulated within intact strips with the process by which sugar is absorbed from the intestine, *in vivo*, or transferred from the mucosal to the serosal sides of other types of *in vitro* preparations. It seems clear from the data presented in Tables V and VI that the accumulation of sugar by the intact strip is a property of the mucosal epithelial cells and that sugar appears in the underlying layers of the intestinal wall as the result of diffusion from the high concentration provided by the activity of the epithelial cells or by entrainment in the water stream[40]. Mucosal sheets and villi accumulate sugar to high relative concentrations—serosal sheets do not—and there is no reason to doubt that these studies on accumulation measure the same process that is measured *in vivo* and by other techniques, *in vitro*. By way of contrast, this is not so clearly the case with amino acids. Amino acids have been shown to be transported against an apparent concentration gradient by everted sacs of intestine[50, 51] and it has seemed reasonable to assume that this is a property of the epithelial cells. However, accumulation of amino acids is a property of many cells[52, 53] and the data presented in Table VI show that the intestinal musculature also possesses this property. These results would seem to leave in doubt the precise relationship of experiments on amino acid transport with excised intestinal tissue to amino acid absorption *in vivo*.

ACKNOWLEDGEMENTS

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STUDIES ON THE MECHANISM OF THE INTESTINAL ABSORPTION OF SUGARS

III. MUTUAL INHIBITION, *IN VITRO*, BETWEEN SOME ACTIVELY TRANSPORTED SUGARS*

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SUMMARY

Mutual inhibition of intestinal active transport *in vitro* between glucose and galactose, glucose and 1,5-anhydro-D-glucitol, galactose and 1,5-anhydro-D-glucitol, glucose and 6-deoxyglucose and 6-deoxyglucose and 1,5-anhydro-D-glucitol has been observed. The data provide evidence that is consistent with the assumption that intestinal active transport of sugars is mediated by a single common process.

INTRODUCTION

Recent studies on the specificity of intestinal absorption of sugars[1–4] have brought to fourteen the total number of sugars and related compounds known to be transported against an apparent concentration gradient by preparations of hamster intestine, *in vitro*. A comparison of the structures of these compounds** shows that they possess in common a pyran ring, a methyl or substituted methyl group at carbon-5 of the ring and a hydroxyl group in the glucose configuration at carbon-2. Thus, it has been concluded[2] that these three features are the minimal structural requirements for intestinal active transport. Compounds such as mannose, fructose and the pentoses which are not transported against a concentration gradient lack one or more of these structural features.

The question of next importance is whether all fourteen of the actively transported compounds are transported by the same process or whether there are several processes each of which transports only one or a few of these compounds and which provide,

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[*] Although numbers were not assigned to them when they were published, references 1 and 3 describe experiments on the mechanism of the intestinal absorption of sugars and should be considered to be papers number one and two, respectively, of this series. A preliminary report of portions of this work was made at the Forty-third Annual Meeting of the American Society of Biological Chemists held at Atlantic City, New Jersey, April 13 to 17, 1959.

[**] These compounds are D-glucose, 1,5-anhydro-D-glucitol, 2-C-hydroxymethyl-D-glucose, D-glucoheptulose, 3-O-methyl-D-glucose, 3-deoxy-D-glucose, D-galactose, 4-O-methyl-D-galactose, D-allose, 6-deoxy-D-glucose, 6-deoxy-D-galactose, 6-deoxy-6-fluoro-D-glucose, 7-deoxy-D-glucoheptose and α-methyl-D-glucoside.

collectively, the observed overall specificity. An answer to this question would materially aid studies of the mechanism of intestinal absorption. If there is only one process, a number of possible endergonic reactions of sugars may be excluded from participation in the process on the basis of which functional groups of the sugar molecule are not essential for transport[1,3]. The present report provides evidence, in terms of mutual inhibition between some of the actively transported compounds, that is consistent with a conclusion that all of them are transported by a single process.

MATERIALS AND METHODS

The present experiments were carried out by the intact strip technique using the materials and methods described in the preceding paper[5]. The volume of medium, the sugar concentrations and the duration of incubation were varied as noted below. The incubation temperature was 37°.

EXPERIMENTAL

Determination of apparent K_m *values*

Transport of sugar against an apparent concentration gradient by *in vitro* preparations of small intestine exhibits MICHAELIS-MENTON[6] kinetics; that is, the rate of transport increases with sugar concentration in the manner predicted by this theory. In previous studies, apparent K_m values were determined for glucose with rat[7] and guinea pig[8] intestine, for galactose with rat[9] intestine and for 1,5-anhydro-D-glucitol and 6-deoxy-D-glucose with hamster[10] intestine. In the present studies, the K_m values have been redetermined not only because of possible species variation but also because of differences in the way the transport rate is measured. In the previous studies, transport was measured by the movement of sugar through the entire intestinal wall from the mucosal to the serosal surface. In the present studies, transport is measured by the accumulation of sugar within strips of tissue. Thus, the barrier to diffusion presented by the tissues underlying the epithelial cells has been eliminated as a factor in the measured rate and in the apparent K_m (see ref. 5). The K_m values found with the present method are significantly different from those found with other *in vitro* methods.

Apparent K_m values for glucose, galactose and 1,5 anhydro-D-glucitol were determined. The data from which these were calculated are presented in a linear form[11] of the MICHAELIS-MENTEN equation[6] in Figs. 1 and 2. Two experiments were performed with glucose (curves A and B, Fig. 1) each of them designed to minimize the influence of utilization on medium and tissue sugar concentrations, but in different ways. In Expt. A, the volume of the incubation medium was adjusted to provide approximately the same total amount of glucose at each concentration level. The volumes used ranged from 640 ml at the lowest concentration to 20 ml at the highest. Since the amount of tissue in each vessel was about the same, the proportions of total glucose utilized were nearly equal. In Expt. B, advantage was taken of the fact that in a medium free of calcium and magnesium ions, the addition of 0.02 M NaF resulted in 85 % inhibition of glucose utilization with only 15 % inhibition of the rate of glucose accumulation. The K_m values obtained in these two experiments did not differ greatly (see legend to Fig. 1) and the average of them, $K_m = 1.5$ mM, was taken as the apparent K_m for glucose with intact strips.

The experiment with D-galactose, curve C in Fig. 1, was set up like Expt. A, above. The volumes of medium ranged from 50 ml at the lowest concentration of galactose to 10 ml at the highest. The experiment with 1,5-anhydro-D-glucitol (Fig. 2), on the other hand, did not require precautions of this sort inasmuch as utilization of

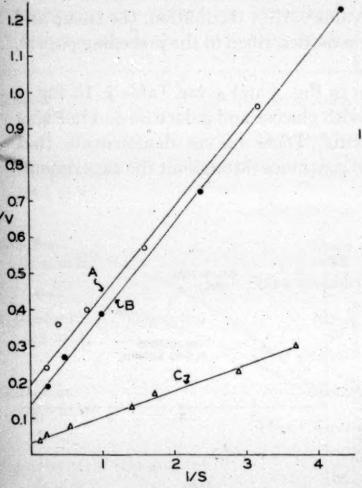


Fig. 1. Relationship of the rate of sugar active transport by intact strips to the external concentration of sugar. Expt. A: Glucose. Incubation was for 8 min. The extrapolated value for K_m = 1.25 mM. Expt. B: Glucose. Calcium and magnesium ions were omitted from the buffer. NaF, 0.02 M was added. Incubation was for 10 min. The extrapolated value for K_m = 1.8 mM. Expt. C: Galactose. Incubation was for 10 min. The extrapolated value for K_m = 2.2 mM. For further experimental details, see the text.

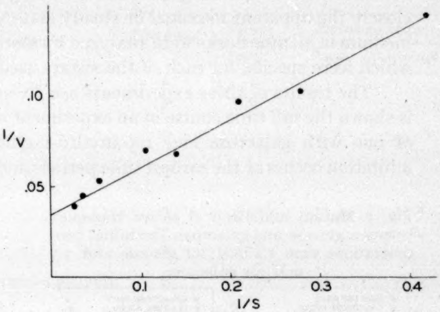


Fig. 2. Relationship of the rate of active transport of 1,5-anhydro-D-glucitol by intact strips to the external concentration. S = the initial medium concentration, V = the final tissue concentration. Incubation was for 10 min. The extrapolated value for K_m = 7.4 mM.

1,5-anhydro-D-glucitol was barely measureable. Utilization of this compound does not proceed beyond phosphorylation by hexokinase and the ester which accumulates inhibits the hexokinase reaction[12]. This ester was not included in the analyses of tissue concentrations inasmuch as it was removed in the deproteinization procedure employing barium hydroxide and zinc sulfate[5]. The K_m value for galactose was 2.2 mM and for 1,5-anhydro-D-glucitol, 7.4 mM.

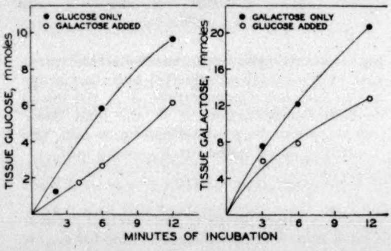
In these experiments the velocity, v, was taken as equal to the final tissue concentration. Thus, the values do not represent initial rates, as would be desirable. It has been assumed that the same proportionality exists between these rates as would have been found between initial rates.

Mutual inhibition between actively transported sugars

Mutual inhibition of transport was studied with the following pairs of sugars: glucose and galactose, glucose and 1,5-anhydro-D-glucitol, galactose and 1,5-anhydro-D-glucitol, glucose and 6-dexoyglucose and 6-deoxyglucose and 1,5-anhydro-D-glucitol. Experiments were designed in the following way: Each sugar was used at a single concentration which was chosen on the basis of the relative K_m values. Intact strips from the small intestine of 3 or 4 fasted animals were divided among nine flasks. One sugar of a pair was added to three of the flasks, the other sugar to another three, and both sugars to the remaining three. Periods of incubation were selected, on the basis of preliminary experiments, with the view to obtaining demonstrable tissue accumulation of both sugars without having the tissue concentration approach too closely the apparent maximal or steady state value[5]. After incubation, the tissue and medium in all nine flasks were analyzed by methods, described in the preceding paper[5], which were specific for each of the sugars used.

The results of these experiments are given in Fig. 3 and 4 and Table I. In Fig. 3 is shown the full time course of an experiment with glucose and galactose and in Fig. 4 of one with galactose and 1,5-anydro-D-glucitol. These curves demonstrate that inhibition occurs at the earliest time periods and continues throughout the experiment.

Fig. 3. Mutual inhibition of active transport between glucose and galactose. The initial concentrations were 1.1 mM for glucose and 5.5 mM for galactose.



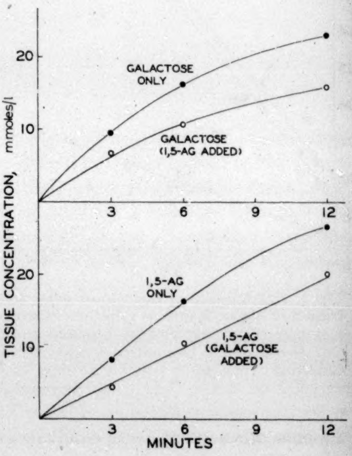


Fig. 4. Mutual inhibition of active transport between galactose and 1,5-anhydro-D-glucitol. The initial external concentrations were 3.8 mM for galactose and 34 mM for 1,5-anhydro-D-glutitol.

In many experiments, however, the degree of inhibition changed throughout the time course. For example, the inhibition of glucose by another sugar was always found to be less at the later time periods. One reason for this seems to be the fact that nearly the same maximal level of glucose was attained in the presence of the second sugar as in its absence. However, there also appears to have been an influence of accumulated tissue glucose on the accumulation of the second sugar so that the degree to which the second sugar was inhibited increased with time, as shown for galactose in Fig. 3. An explanation for this phenomenon is not available in the absence of a knowledge of the mechanism of active transport.

The data in Table I show that mutual inhibition occurred with all pairs of sugars tested. Because of the changing degree of inhibition with time, the earliest time period was chosen in each instance for the purpose of calculation. Where K_m values determined by the intact strip technique were available, the theoretical degree of inhibition expected under the conditions of the experiment was also calculated. For the pairs, glucose and galactose and glucose and 1,5-anhydro-D-glucitol the inhibition found

TABLE I

MUTUAL INHIBITION BETWEEN VARIOUS PAIRS OF SUGARS

The initial external concentrations are given in parentheses below the name of the sugar. All data are taken from experiments designed as described in the text. Curves for the full time period (8 to 12 min) were obtained and were similar to the curves shown in Figs. 3 and 4. The methods of assay are described in the preceding paper[5].

Test compound	*Compound added*	*Time period (min)*	*Tissue concentration (mM)*	*Per cent inhibition*	
				Found	*Calculated*
Glucose (1.1 m*M*)	None	3	3.0		
	Plus galactose	3	1.25	58.5	59.0
[1-^{14}C]galactose (5.5 m*M*)	None	3	7.5		
	Plus glucose	3	5.9	21.4	15.8
Glucose (1.0 m*M*)	None	2	2.76		
	Plus 1,5-anhydro-D-glucitol	2	1.67	44	63.5
1,5-anhydro-D-[^{3}H]glucitol (25 m*M*)	None	2	2.4		
	Plus glucose	2	1.0	58	61.5
Galactose (3.8 m*M*)	None	3	9.5		
	Plus 1,5-anhydro-D-glucitol	3	6.4	32.6	63
1,5-anhydro-D-[^{3}H]glucitol (34 m*M*)	None	3	8.2		
	Plus galactose	3	5.1	37.8	19.5
Glucose (0.86 m*M*)	None	6	4.2		
	Plus 6-deoxyglucose	6	3.1	26	—
6-[^{3}H]deoxyglucose (0.86 m*M*)	None	6	2.0		
	Plus glucose	6	1.7	15	—
1,5-anhydro-D-[^{3}H]glucitol (4.8 m*M*)	None	3	2.7		
	Plus 6-deoxyglucose	3	1.5	44.5	—
6-deoxyglucose (0.48 m*M*)	None	3	1.8		
	Plus 1,5-anhydro-D-glucitol	3	1.3	27.8	—

compared closely with that calculated from the K_m values. With the pair, galactose and 1,5-anhydro-D-glucitol, a significant disparity was found. However, it may be calculated that this disparity is completely accounted for if there is an error in the relative K_m values of galactose and 1,5-anhydro-D-glucitol of about 2.

Inhibition of active transport by other sugars

Active sugar transport was not measureably influenced by the presence of compounds which are not actively transported when the relative concentration of the latter was small. However, when high relative concentrations of the latter were used, some of them caused a significant degree of inhibition. For example, D-xylose at 47 mM inhibited the active transport of glucose, 3 mM, by 15.6 %. 1,5-anhydro-D-mannitol, on the other hand, at 47 mM had no effect. Further experiments were made with 1,5-anhydro-D-glucitol at 5 mM as the test sugar with the addition of non-actively transported sugars at a uniform concentration of 24 mM. The inhibitions observed were D-xylose, 23.4 %, D-ribose, 8.6 %, 2-deoxy-D-galactose, 1.0 % and 6-deoxy-L-galactose, 42.4 %. Collectively, these data indicate that some portion of the process of active transport is susceptible to inhibition by sugars which are not actively transported. There also seems to be some degree of specificity to the inhibition. Further studies along these lines are in progress in an effort to determine fully the specificity of inhibition by non-actively transported compounds. A tentative suggestion that may be made for this inhibition is that these compounds which lack one or more of the structural features necessary for active transport[2] combine with the same site or structure as the actively transported compounds but they cannot undergo the subsequent steps in the process which lead to accumulation against a concentration gradient.

DISCUSSION

Until the full details of the mechanism of the process of active transport of sugars by the small intestine are known, the precise value of studies of mutual inhibition cannot be decided. Were the system under study a simple one, such as purified single enzyme, however, it could be considered proved from the data presented above that only one process is involved in the active transport of all sugars. Although all known actively transported sugars have not been studied, those above, together with 3-O-methyl-D-glucose, reported by CSAKY[13] to inhibit competitively the active transport of glucose, represent a modification at each carbon atom of glucose except, of course, for carbon-2 which is specifically required for active transport[1,2].

ACKNOWLEDGEMENTS

The technical assistance of M. L. ROBERTS is greatly acknowledged. This investigation was supported by Grant No. G-5892 from the National Science Foundation.

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STUDIES ON THE MECHANISM OF INTESTINAL ABSORPTION OF SUGARS

IV. LOCALIZATION OF GALACTOSE CONCENTRATIONS WITHIN THE INTESTINAL WALL DURING ACTIVE TRANSPORT, *IN VITRO**

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SUMMARY

The concentrations of actively transported D-galactose in various parts of frozen, dried sections of hamster intestine have been measured. The relationships of these concentrations strongly indicate that active transport of sugars by the small intestine is mediated by a process which lies in or near the luminal or brush-border end of the epithelial cells.

INTRODUCTION

The mechanism of the process of intestinal active transport which accounts for the absorption of sugars against an apparent concentration gradient is unknown. Recent *in vitro* studies of the chemical aspects of this process, embracing its substrate specificity[1–3] and kinetics[4] and the absence of oxygen exchange reactions between transported sugar and water[5], have eliminated a variety of possible endergonic reactions of sugars as participants. Since they have also failed to uncover direct evidence implicating any specific reaction or reaction sequence[5], there is still no sound basis on which to erect a hypothesis of the chemistry of intestinal active transport of sugars.

There is hope, however, that studies along different lines will yield some insight into the mechanism of the process. For example, with a knowledge of its precise location within the cell, an apparent interdependence or linking of active sugar transport with other localized cellular functions or enzymes could be evaluated on the basis of whether both are contained in the same or different portions of the cell. Since its chemistry is unknown, the process cannot be visualized by the usual histochemical approach. However, as an alternative, the relative concentrations of actively trans-

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Abbreviations: DPN, DPNH, oxydized and reduced diphosphopyridine nucleotide.

[*] A preliminary report of portions of this work was made at the Forty-third Annual Meeting of the American Society of Biological Chemists held at Atlantic City, New Jersey, April 13 to 17, 1959.

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ported sugar in the various cell layers of the intestinal wall can be measured and the probable location of the active transport process can be deduced from their relationships. The present report describes experiments of this kind and provides a partial answer to the question of the localization of the process of active sugar transport. The histologically-distinct cell layers of frozen-dried sections of hamster intestine were analysed by a specific enzymic assay for D-galactose[6] which was sensitive enough to measure quantitatively as little as 10^{-11} moles of sugar contained in a tissue sample weighing 0.1 μg or less. The sugar accumulated during incubation with D-galactose, *in vitro*, was found to be present, at the earliest time periods and at the highest concentrations, within the epithelial cell layer.

MATERIALS AND METHODS

Intact strips of hamster intestine were prepared and incubated in the presence of D-galactose by methods that have been previously described[7]. The concentration of galactose was 5 mM; the temperature of incubation was 37°. At intervals during incubation, sample strips were removed from the flasks. Some of them were treated and analysed, *in toto*, as previously described[7]. Others were blotted gently and plunged into dichlorodifluoromethane at its freezing point (—160°). From the latter, frozen-dried sections, 15 μ to 40 μ in thickness, were prepared by the methods described by LOWRY[8]. The various cell layers of these sections were easily distinguished under low magnification with an ordinary microscope, as shown in Plate 1. Selected portions of the epithelial cell layer, the tunica propria ("core") and the underlying tissue layers were dissected and weighed, and their volume was determined[8]. The crypt epithelium was not used and only that portion of the tunica propria which extends from the level of the orifices of the crypts of Lieberkuhn to the tips of the villi was used. The weight of the tissue samples used ranged from 0.02 to 0.14 μg, corresponding to volumes ranging from 10^{-4} to 10^{-3} μl. In order to calculate tissue

Plate 1. Photomicrograph of frozen, dried section of hamster intestine. Total magnification 60 ×.

water from the measured dry weight and tissue volume, the density of the frozen-dried material was assumed to be 1.3*.

D-galactose present in the frozen, dried tissue samples was measured by means of the reaction catalyzed by the galactose dehydrogenase of *Pseudomonas saccharophilia*[9]. This enzyme, which was used in a partially purified form (30–40 fold**), catalyses a DPN-dependent oxidation of D-galactose to D-galactonolactone, with the stoichiometric reduction of DPN[9]. The DPNH formed during incubation of the tissue samples with the enzyme and 0.05 m*M* DPN for 2 h under conditons described elsewhere[6] was measured fluorometrically[11]. Control analyses showed that 10 $\mu\mu$moles of galactose produced consistently 90 to 100 % of the theoretical amount of DPNH. The techniques and precautions used followed the general methods for analysis at this level as described by LOWRY *et al.*[12, 13].

Tests of the specificity of the enzyme showed it to the adequate for the present purpose. The preparation showed moderate activity toward L-arabinose, but none toward D-arabinose, D-ribose, L-galactose, D-glucose and D-mannose. In tests with D-galactosamine** and D-galactose 1-phosphate***, an amount of DPNH equivalent to 3 % of the former and 6 % of the latter was formed during two hours of incubation.

EXPERIMENTAL

The results of these studies are presented in Table I and Fig. 1. The analyses in Table I were made of tissue that had been incubated for the single time period of 15 min. After incubation, the tissue was recovered and processed as described in the preceding section. Assays for galactose were made on the total tissue and on four readily distinguished portions of the frozen, dried sections; the epithelial cell layer, the tunica propria or "core" of the villi, the submucosa and the muscularis (see Plate 1). In Expt. 1, Table I, the data in the column to the far right show that the highest concentration of galactose was present in the epithelial cell layer. In Expt. 2, Table I, analytical data are given for epithelium and for "peeled" epithelium. This experiment was done to provide assurance that the higher concentration found in the epithelial cell layer was not caused by the presence of dried, galactose-containing medium on the surface of the tissue. When the outermost or luminal 5 to 10 μ portion of the epithelial layer was cut off before analysis, the concentration found was somewhat less than when this operation was not done. However, the concentration in the "peeled" epithelium was still much higher than the concentration in the "core". Expt. 3, Table I, was carried out for much the same purpose. In this experiment, samples of tissue were taken at the end of 15 min incubation and processed directly. Other samples were taken and transferred to a galactose-free medium for an additional one minute period of incubation. These were then recovered and processed. The concentration difference between the epithelial cell layer and the "core" was maintained during this "washing" period.

The curves in Fig. 1 show the results of analyses performed on tissue recovered

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* Based on an assumed tissue content of fat = 5 % of wet weight, density, 0.9; protein = 15 %, density, 1.35; and salts = 1.5 %, density, 2.34 (for KH_2PO_4).

** We are indebted to Dr. M. DOUDOROFF for a sample of the enzyme, some of the organisms, and for instructions for preparing the enzyme[10], in advance of publication.

*** We are indebted to Dr. J. L. STROMINGER for samples of these compounds. Free galactose was a possible small contaminant of both.

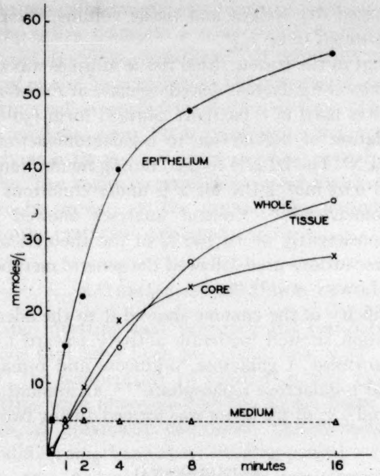


Fig. 1. Time course of the accumulation of D-galactose in intact strips of hamster intestine during incubation at 37°.

TABLE I

CONCENTRATIONS OF D-GALACTOSE FOUND IN VARIOUS PARTS OF INTACT STRIPS OF HAMSTER INTESTINE

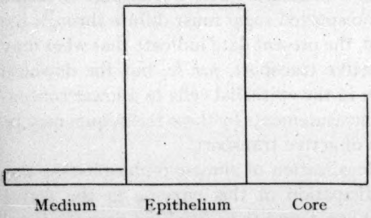
Incubated for 15 min at 37° in 5 m*M* galactose. The numbers in parentheses indicate the number of tissue samples assayed. Standard errors are indicated. The concentration in the total tissue was determined by methods previously described[7]. The concentrations in the various tissue layers were determined on frozen, dried material as described in the present paper. The convention of reporting tissue analyses on a kg basis originates with Lowry[8]. For further details consult the text.

Expt.	*Tissue sample*	*Water content l/kg dry wt.*	*Galactose concentrations*	
			mmole/kg dry wt.	*mmole/l H_2O*
I	Epithelium (8)	4.30 ± 0.26 (6)	211 ± 8	49.1 ± 2.1
	Core (10)	7.03 ± 0.36	199 ± 13	28.3 ± 1.9
	Submucosa (7)	10.52 ± 0.62	292 ± 20	27.8 ± 1.9
	Muscularis (8)	3.69 ± 0.12 (7)	72.7 ± 3.0	19.6 ± 0.91
	Total tissue			38.4
II	Epithelium (9)	3.26 ± 0.8 (8)	112.4 ± 5.7	34.5 ± 1.8
	Epithelium (peeled) (4)	—	90.0 ± 3.1	27.6 ± 0.95
	Core (8)	4.76 ± 0.29	83.2 ± 1.7	17.5 ± 0.36
III		Before washing		
	Epithelium (8)	5.95 (7)	160	28.0 ± 2.3
	Core (8)	7.51 (6)	123	14.4 ± 1.8
	Total tissue			18.9
		After washing		
	Epithelium (10)	5.84 (6)	150	26.5 ± 2.7
	Core (8)	7.39 (5)	128	17.5 ± 1.9
	Total tissue			17.9

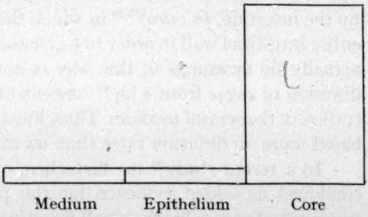
at various time intervals during incubation. At the earliest time period, 15 sec., the epithelial cells contained galactose at a concentration at least equal to that of the medium. At the 1-min time period, the epithelial cells contained three times the medium concentration. At these same time intervals, on the other hand, the concentration in the "core" of the villi lagged greatly behind the concentration in the epithelial cells. At the 1-min period, for example, the concentration in the epithelial cells was 3 times the concentration in the "core". Throughout the entire 16 min of observations, a large concentration difference between epithelial cells and "core" was maintained, indicating that the rate of active transport into the epithelial cells from the medium was much faster than diffusion into the underlying tissues. The initial rate of galactose active transport extrapolated from the value at the 15 sec interval was 1080 μmoles/g wet wt./h (4.2 moles/kg dry wt./h).

DISCUSSION

The major possibilities with respect to the relative concentrations of galactose present in the medium, the epithelial cells and the "core" of the villi after incubation are shown in the following diagrams.



A



B

The relationship depicted in diagram A is what would be expected if the process mediating active sugar transport lies in or near the luminal or brush-border pole of the epithelial cells and if most of the cells have this activity. If, on the other hand, the process were localized at the basal pole of the epithelial cells and most of the cells were active, the relationship shown in diagram B would be expected. If only a few of the cells were active in transporting galactose or if the rate of diffusion of sugar between the epithelial cells and the "core" were as fast or faster than the rate of active transport, one would expect the concentrations in the epithelial cells and the "core" to be nearly equal.

The relationship of galactose concentrations shown in diagram A was found in the present study. Galactose was accumulated against an apparent concentration gradient by the epithelial cells and at all time periods during incubation the concentration of galactose in the epithelial cells was markedly higher than in the immediately adjacent "core" of the villi. This is strong support for the conclusion that active transport of sugars by the small intestine is mediated by a process which lies in or near the luminal pole of the epithelial cells. It is also evidence contrary to the suggestion made several years ago[14] that active transport of sugars is a property of a special kind of epithelial cell which is present in relatively small numbers.

Whether the galactose accumulated within the epithelial cells is in a "free" state cannot be decided from the data available. The manipulations of the tissue prior to and during analysis for galactose do not include any procedures which could be expected to rupture covalent bonds. Thus, if binding of galactose to an intracellular component occurs, it is most likely a freely reversible association. Assuming galactose to be in the "free" state within the cells, the concentration relationships between the epithelial cells and the medium are such as to suggest either that there is a large osmotic pressure difference between the two, that another solute has leaked or been "pumped" from the epithelial cells into the medium to prevent this pressure difference, or that water has entered the cells with the sugar. The recent observations that active transport of sugars requires the presence of Na^+ in the medium[15,16] and that the steady state level of sugar accumulation is dependent upon Na^+ concentration* suggest that ion "pumping" is directly involved in sugar transport. Also our data suggest that some water moves into the cells during sugar transport. However, these data are inadequate to show the exact change in cell volume and are not included in the present paper. In the absence of solute exchange between the intracellular and extracellular compartments, osmotic equilibrium at the 16-min interval in Fig. 1 would require an increase in cell volume of 20 %.

With respect to the various methods used to measure active transport of sugars by the intestine, *in vitro*[17-19] in which the transported sugar must diffuse through the entire intestinal wall in order to be measured, the present data indicate that what may actually be measured in this way is not active transport, *per se*, but the downhill diffusion of sugar from a high concentration in the epithelial cells to a lower concentration in the serosal medium. Thus, kinetic measurements by these techniques may be based more on diffusion rates than on rates of active transport.

In a recent study[20] the histochemical localization of glucose-6-phosphatase was employed as added evidence for the participation of this enzyme in the active transport of sugars by the small intestine. It was found to be localized near the basal pole of the epithelial cells from which point the sugar released from glucose-6-phosphate or other primary phosphate esters[21] could then diffuse into the core of the villus and into the blood stream. The present data indicate that this enzymic activity is inappropriately localized for participation in the events of active sugar transport.

ACKNOWLEDGEMENTS

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GALACTOSE 1-PHOSPHATE IN THE INTESTINAL TISSUE OF THE RAT DURING GALACTOSE ABSORPTION

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SUMMARY

The water-soluble, ethanol-insoluble barium salt mixture from a trichloracetic acid extract of the small intestines of rats assimilating galactose was resolved into several fractions on a column of Dowex-1 borate. Each of the fractions consists of several components. Galactose was identified in three of the fractions, including one in which acid-labile phosphates predominated. An enzymic method was used to confirm the presence of galactose 1-phosphate in similar fractions from intestinal extracts which had not been treated with barium. A high level of galactose 1-phosphate appears in the intestinal tissue during the absorption of galactose, but none is present when sorbose is being absorbed. Raising the rats on diets containing low levels of galactose or lactose does not influence the level of intestinal galactose 1-phosphate, measured 1 h after the administration of galactose by stomach tube. The galactose 1-phosphate is formed by the phosphorylation of free galactose in the intestinal tissue.

INTRODUCTION

In spite of the interest that attaches to a knowledge of the metabolism of galactose in intestinal tissue, it has not previously been established whether this tissue has the ability to phosphorylate galactose. HELE[1], SOLS[2], and others[3] have tested homogenates of intestinal tissue for galactokinase activity, but have obtained conflicting results. Other investigators have sought galactose phosphates in the tissue. KJERULF-JENSEN[4] found that the "hexose monophosphate fraction" from the intestinal mucosa of rats which were assimilating galactose gave a strong phloroglucinol test, and since the fraction had a high reducing power and little acid-labile organic phosphate, he suggested that galactose 6-phosphate might be present. More recently, NAGASAWA[5] administered $NaH_2{}^{32}PO_4$ to rats along with galactose, and from the intestines obtained radioactive compounds with the paper chromatographic behavior expected of galactose 1- and 6-phosphates. However, neither author completed the identification of his supposed galactose esters. A careful examination of the sugar phosphates

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Abbreviations: TPN and TPNH, triphosphopyridine nucleotide and its reduced form; UDP-glucose, uridine diphosphate glucose; PGM, phosphoglucomutase.

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[**] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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formed in rat intestinal tissue during galctose absorption, with the aim of positively identifying any galactose phosphates present, was therefore needed. Such an investigation is the principal subject of this paper.

A second subject is the hexose phosphate content of rat intestinal tissue. It has been known for two decades that, during the absorption of the common, well metabolized hexoses, the amounts of acid-soluble phosphates in the intestinal mucosa increase[6–8]. The evidence indicated that the extra phosphates consist at least in part of hexose phosphates. But the difficult (at the time) job of identifying the individual phosphates was not undertaken by the previous investigators. Hence, no estimates were available of the quantities of individual esters present. In the present work, the aldose 1-phosphates were determined in intestinal tissues from rats which were assimilating the "metabolically inert" sugar L-sorbose, and from those which were assimilating D-galactose. In connection with these determinations, an effort was made to detect any possible metabolic adaptation, involving galactose 1-phosphate levels in the intestine, which might result from the addition of low and non-toxic levels of galactose and lactose to the diet.

MATERIALS AND METHODS

Sugar phosphates

Barium galactose-1-phosphate was synthesized according to the directions of HANSEN *et al.*[9]. Dr. H. A. LARDY generously provided samples of dipotassium glucose 1-phosphate and barium galactose and mannose 6-phosphates. Barium glucose and fructose 6-phosphates were commercial preparations.

Coenzymes

TPN-sodium (96–98 %) and UDP-glucose sodium (90 %) were obtained from the Sigma Chemical Co., St. Louis.

Enzymes, etc.

Acid phosphatase (wheat germ) was purchased from the Mann Research Laboratories. The glucose 6-phosphate dehydrogenase routinely used was Zwischenferment, Practical, Type II, from the Sigma Chemical Co. Phosphoglucomutase was prepared from rabbit muscle by the procedure of NAJJAR[10]. The crystalline protein which was obtained reverted to amorphous form, but it retained high activity. Galactose 1-phosphate uridyl transferase was prepared according to KURAHASHI AND ANDERSON[11].

Rats were obtained from the Holtzman Animal Laboratories, Madison.

Ion exchange chromatography was carried out on columns of Dowex-1 borate as described by DIEDRICH[12]. The basic procedure is a modification of that of GOODMAN, BENSON AND CALVIN[13].

Hexose analyses

The anthrone method of ROE[14] was employed for the quantitative determination of the hexose content of pooled fractions, etc. This method was also used initially for the detection of chromatographic peaks. Later, a more sensitive and selective colorimetric method for hexoses, developed in this laboratory[12], was used for monitoring the effluents from the ion exchange columns.

Phosphate analyses

Phosphate analyses were carried out by the Fiske and SubbaRow method[15]. When hexose-1-phosphates were present, samples for the inorganic phosphate determination were diluted to 9 ml with water and cooled to 20° before the acid molybdate and reducing agent were added. By letting the color development proceed at 20 ± 1° (for exactly 15 min) the hydrolysis of hexose 1-phosphate was held to a minimum (0.4 % for glucose 1-phosphate)[16]. Samples for the total phosphate determination were first digested with nitric acid, then perchloric acid. After dilution with water and boiling to hydrolyze pyrophosphate, the samples were neutralized with NaOH and analyzed for inorganic phosphate.

The enzymic assay of hexose 1-phosphates is discussed in the EXPERIMENTAL section.

Preparation of intestinal extracts

The entire procedure was carried out in a cold room, and cold solutions were used. The animal was killed by a blow on the head, decapitated, and exsanguinated. The small intestine was cut at the pyloric sphincter and pulled free of the adhering mesentery, and the upper two thirds was taken for extraction. Isotonic NaCl was pumped into the duodenal end of the dissected organ from a wash bottle, with momentary clamping of the ileal end to cause distension, and the exterior was rinsed in a beaker of saline. Excess saline was blotted off, the intestine was weighed to the nearest 0.1 g, cut into strips 2–3 cm long, and homogenized in 20 ml of 10 % trichloracetic acid in a small Waring blendor. The homogenate was transferred to a plastic tube with distilled water and centrifuged, and the precipitate washed once with 20 ml of 5 % trichloracetic acid. The combined supernatants were kept frozen at −10°. When further workup was to be started, the frozen solutions were thawed overnight at *ca* 10°, filtered through glass wool, and extracted three times with their own volume of ether to remove trichloracetic acid and lipids. The solutions were then neutralized and the dissolved ether was flashed off *in vacuo*.

EXPERIMENTAL

Identification of galactose 1-phosphate in intestinal tissue

By ion exchange chromatography: A number of preliminary experiments were conducted in which a single rat was allowed to assimilate a dose of galactose for 1 h, and the intestinal extract was subjected to ion exchange chromatography. Sugar phosphate appeared in the eluates in the position corresponding to galactose 1-phosphate, but the amounts were too small for further characterization. It was therefore necessary to pool the extracts from a number of intestines, even though the pooled extracts could not be chromatographed directly. The large amount of inorganic salts present would have made a very large column necessary.

Forty adult female rats were fasted for 10–15 h, then each rat was given 4 ml of 0.75 *M* D-galactose by gavage. Extracts were prepared from the intestines and pooled, and the resulting solution, free of trichloracetic acid, was neutralized and concentrated *in vacuo* to a volume of 100 ml. The water-soluble, ethanol-insoluble barium salt fraction was prepared from the concentrate in the usual way[17]. Barium was then removed from an aqueous solution of the salts by precipitation with a slight excess of K_2SO_4, and the supernatant was chromatographed on a column of Dowex-1 borate.

This fractionation of pooled extracts from the intestines of a large number of rats was repeated a number of times, with similar results each time. A typical elutogram is shown in Fig. 1, and the information which was obtained about the composition of the peaks is summarized in the following paragraph. In addition to the assays for carbohydrate (anthrone), analyses for acid-labile and acid-stable organic phosphate were performed on the pooled fractions representing each peak. Portions of the material from each peak were also converted to the free sugars for paper chromatographic analysis. Hydrolysis of the acid-labile phosphates was accomplished by making the solutions 1 *N* in HCl and heating at 100° for 10 min. Fractions containing acid-stable phosphate were first freed of cations by passage over Dowex-50 (H^+), then freed of boric acid by extraction with isoamyl alcohol[12]. Wheat germ phosphatase (which is strongly inhibited by boric acid) was used to hydrolyze the phosphates. All hydrolyzates were deionized by successive passages over Dowex-50 (H^+) and Dowex-1 (OH^-), concentrated, and spotted on Whatman No. 1 paper. The solvent mixture of DeWhalley *et al.*[18], *n*-butanol–pyridine–water–benzene (5:3:3:1), was used for the chromatography (descending). The aniline hydrogen phthalate reagent of Partridge[19] and Wolfrom and Miller's[20] reagents for vicinal hydroxyl groups were used (on separate papers) to reveal the spots.

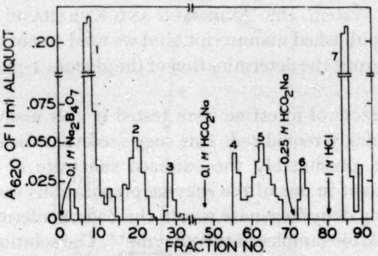

Fig. 1. Chromatography of the water-soluble, ethanol-insoluble barium salt fraction of pooled intestinal extracts. Resin, Dowex-1 X-8, 50–100 mesh, borate form. Column size, 1.2 cm (diameter) × 26 cm. Fraction volume, 40 ml. "A_{620} of 1 ml aliquot" designates the absorbancy, at 620 mμ, found when a 1 ml aliquot was analyzed by the anthrone method. An A_{620} value of 0.1 is given by 22 μg of sugar, calculated as glucose.

In peak 1, the Wolfrom-Miller reagents disclosed at least 6 compounds, including glucose and galactose. Peak 2, in which the phosphate was largely acid-labile, contained galactose and an unidentified sugar. Peak 3 contained both acid-labile and acid-stable phosphates and a number of unidentified compounds which reacted with the Wolfrom-Miller reagents. It was not further studied. Peak 4, in which the phosphate was largely acid-stable, gave a prominent spot with the R_F of galactose. Peak 5 (acid-stable phosphates) yielded sugars with the R_F values of glucose and ribose, and an unidentified sugar. Peak 6 (acid-stable phosphates) gave three spots, one with the R_F of ribose. The latter two peaks had nucleotide-like u.v. absorption spectra. Peak 7 represents the material which was stripped from the column by 1 N HCl. It was not further investigated.

Enzymic identification

In view of the difficulties encountered in the foregoing attempts to resolve the ethanol-insoluble barium salt fraction of intestinal extract by ion exchange chromatography, an enzymic method for the positive identification of galactose 1-phosphate was indicated. Preliminary work was done on a system containing the hexose 1-phosphate epimerizing system of calf liver[21], phosphoglucomutase, and glucose 6-phosphate dehydrogenase (Zwischenferment). This system should convert galactose 1-phosphate to 6-phosphogluconate with the concomitant generation of TPNH. At this time, Dr. ELIZABETH ANDERSON informed us that she and Dr. K. KURAHASHI had already developed a Zwischenferment assay for galactose 1-phosphate, using galactose 1-phosphate uridyl transferase, and UDP-glucose at the substrate level, instead of the complete epimerizing system. Drs. ANDERSON AND KURAHASHI kindly furnished us a copy of their then unpublished manuscript, and we used essentially their procedure[11], slightly modified to permit the determination of the glucose 1-phosphate present in the sample.

When crude extracts of intestine were tested in this assay system, TPNH was formed, but it was quickly reoxidized. This suggested that the extracts contained an interfering substance, presumably the oxidized substrate of some dehydrogenase present as a contaminant in one of the enzyme preparations used. It could be shown that the contaminating dehydrogenase was in the Zwischenferment[*], and that it was present in all the available samples of this enzyme[**]. The solution to the problem thus lay in removing the interfering substrate from the intestinal extracts. Since a simple purification by adsorption on Dowex-1 and elution with formate did not accomplish the desired results, the samples were chromatographed in the Dowex-1 borate system.

The samples were prepared as described above, except that each sample consisted of the crude extract of a single rat intestine, freed of trichloracetic acid, but not fractionated with barium. Samples were adjusted to pH 4 before being put on the column, since the resin does not completely adsorb sugar phosphates from neutral solutions containing large amounts of inorganic salts. The fractions of the eluate (from an individual sample) corresponding to peaks 1 and 2 were pooled and an ion exchange method[12] was used to remove sodium ions and boric acid, which strongly inhibits Zwischenferment. The borate-free eluate, which was now in 0.1 N ammonium formate at pH 6, was concentrated from the frozen state to a small volume, made up to 10 ml, and an aliquot was taken for enzymic assay. The addition of phosphoglucomutase gave an increment of TPNH, as expected for samples containing glucose 1-phosphate. In samples from rats which had received a dose of galactose 1 h before the intestines were removed, the addition of galactose 1-phosphate uridyl transferase gave a second increment of TPNH, as expected for samples containing galactose 1-phosphate. Fig. 2 records the results of a typical experiment.

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[*] It is quite likely that the interfering substrate was oxidized glutathione. Glutathione reductase was found[22] in samples of Sigma Zwischenferment which were on the market when the present work was being carried out.

[**] We thank Dr. S. A. KUBY of the Institute for Enzyme Research, Madison, for a sample of Zwischenferment prepared by an unpublished procedure.

Quantitative determination of hexose 1-phosphates in rat intestine

Forty weanling, female rats were divided into four groups of ten. The animals were maintained for 30 days on diets consisting of a standard stock ration containing no supplement, 8 % D-glucose, 8 % D-galactose, and 16 % lactose, respectively. Diet and water were given *ad libitum*, and each animal received a dose of Haliver oil weekly. Within experimental error, the average gains of the animals in the different groups were identical and none showed any toxic effects. The average weight achieved was 125 g.

At the end of the 30 day period, the animals were fasted 10–15 h. Five animals from each group received 4 ml of 0.75 *M* L-sorbose by stomach tube, and the remaining five animals received 4 ml of 0.75 *M* D-galactose by the same route. One hour after the sugar was administered, the animals were killed and the intestines were extracted as described under MATERIALS AND METHODS. The individual extracts were then chromatographed and assayed enzymically for glucose 1-phosphate and galactose 1-phosphate as described in the preceding section. When known amounts of the hexose 1-phosphates were added to crude intestinal extracts, the procedure gave recoveries of 90 % for glucose 1-phosphate and 60 % for galactose 1-phosphate.

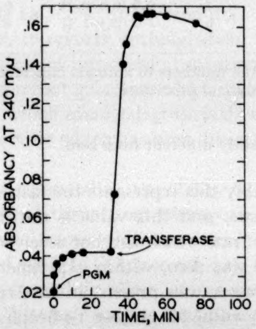

Fig. 2. TPNH production by glucose 1-phosphate and galactose 1-phosphate in intestinal extract. The incubation mixture contained: 0.17 *M* glycylglycine buffer, pH 8.7, 0.6 ml; 0.03 *M* disodium ethylenediaminetetraacetate, 0.1 ml; 0.3 *M* $MgCl_2$, 0.1 ml; 0.15 *M* cysteine at pH 8.5, 0.2 ml; 0.00125 *M* TPN, 0.2 ml; 0.0015 *M* UDP-glucose, 0.1 ml; Zwischenferment, 1 Kornberg unit/ml, 0.1 ml; and water and/or sample, 1.4 ml. Total volume, 2.8 ml. Enzyme additions (0.1 ml each) were: phosphoglucomutase, 0.5 mg protein/ml; and galactose 1-phosphate uridyl transferase, 12.5 mg of lyophilized powder/ml of 0.001 *M* phosphate buffer, pH 6.0. Absorbancy was read in an unthermostated Beckman model DU spectrophotometer.

Prior to the finding that the sugar phosphates of intestinal extracts must be purified chromatographically before they can be assayed in the Zwischenferment system, it was hoped that glucose 6-phosphate could be determined, along with the 1-phosphates. This idea was given up because the continuation of the chromatography until glucose 6-phosphate is eluted is laborious, and there is no assurance that the interfering substrate mentioned above would be separated from the glucose 6-phosphate.

The results of the assays are shown in Table I. It may be seen that the intestines of the animals which received sorbose had a low but readily measurable level of

TABLE I

HEXOSE 1-PHOSPHATE LEVELS IN RAT INTESTINAL TISSUE DURING SUGAR ABSORPTION

Details of the analytical method are given under Fig. 2.

Diet supplement	*Sugar administered*	*No. of animals*	*Hexose 1-phosphate (µmoles/100 g fresh tissue)*[*]		
			Glucose 1-phosphate found	*Galactose 1-phosphate found*	*Galactose 1-phosphate corrected*[**]
None	Galactose	5	0.9 (0.8–1.0)[***]	14 (11–19)	23 (18–32)
8 % glucose	Galactose	3	1.1 (0.9–1.2)	11 (8–15)	18 (13–25)
8 % galactose	Galactose	3	1.4 (1.3–1.5)	14 (10–17)	23 (17–28)
16 % lactose	Galactose	4	0.6 (0.4–1.0)	6 (2–13)	10 (3–22)
All galactose groups		15	1.0 (0.4–1.5)[§]	11 (2–19)	18 (3–32)
None	Sorbose	2	0.8 (0.8–0.9)	0.2 (0.1–0.2)[§§]	—
8 % glucose	Sorbose	3	0.9 (0.3–1.4)	0.0 (0.0–0.1)	—
8 % galactose	Sorbose	2	1.4 (1.3–1.5)	0.0 (0.0)	—
16 % lactose	Sorbose	2	0.6 (0.6–0.7)	0.0 (0.0–0.1)	—
All sorbose groups		9	1.0 (0.3–1.5)	0.0 (0.0–0.2)	—

[*] Figures given are means for the numbers of animals indicated, and (in parentheses) ranges.
[**] For 60 % recovery in the analytical procedure.
[***] 4 values.
[§] 14 values.
[§§] Value not considered significantly different from zero.

glucose 1-phosphate; presumably this represents the fasting level. The average value is 1.0 µmole/100 g of wet tissue, and this value is not increased during galactose absorption. In the intestines of rats which had not received galactose, the concentration of galactose 1-phosphate was zero, within experimental error. But during the absorption of galactose, the ester was present in intestinal tissue in considerable concentration (eleven times the value for glucose 1-phosphate or eighteen times, if the galactose 1-phosphate values are corrected).

In order to rule out the possibility that the galactose 1-phosphate found in rat intestinal tissue is formed in some other organ and brought to the intestine in the blood stream, blood plasma was assayed. Neither galactose 1-phosphate nor glucose 1-phosphate could be detected in a sample of plasma from animals assimilating galactose.

DISCUSSION

From the evidence presented in the foregoing paragraphs, it seems conclusively established that galactose 1-phosphate is formed during the intestinal absorption of galactose in the rat. Galactose 1-phosphate gives a characteristic peak on chromatography in the Dowex-1 borate system. When extracts were made from the intestines of rats assimilating galactose, and the water-soluble, alcohol-insoluble barium salt fraction of the pooled extracts chromatographed in this system, the corresponding peak contained acid-labile organic phosphate and, after hydrolysis, a sugar with the R_F of galactose.

The peaks from the Dowex column are clearly mixtures, but the presence of both glucose 1-phosphate and galactose 1-phosphate in eluates from single intestinal extracts which had not been fractionated with barium and alcohol was confirmed by the response which these eluates gave in the enzymic assay. Reduction of TPN was observed when phosphoglucomutase was added to the system, and again when galactose 1-phosphate uridyl transferase was added. The first rise in absorbancy is attributed to glucose 1-phosphate and the second to galactose 1-phosphate. Phosphoglucomutase, galactose 1-phosphate uridyl transferase, and Zwischenferment are quite specific, and even though the enzyme preparations employed were not pure, it is highly unlikely that the observed responses could be given by substances other than the two hexose 1-phosphates, particularly in a sample which had been subjected to ion exchange chromatography. The fact that a response for galactose 1-phosphate is obtained only in extracts from rats which are assimilating galactose is an additional argument in support of this conclusion. And since the blood plasma is free of galactose 1-phosphate (and glucose 1-phosphate), it may be further concluded that the galactose ester is formed by the phosphorylation of free galactose in the intestinal tissue.

It now becomes of interest to examine the physiological significance of the intestinal phosphorylation of galactose. However, the fact that galactose 1-phosphate appears in the tissue during galactose absorption does not of itself answer questions of this kind. In particular, it reveals nothing about the possible participation of phosphorylation in the process of intestinal absorption. The ester which is found in the tissue could, *a priori*, represent galactose which was in transit when the tissue was excised, or a *cul de sac* into which some galactose is diverted when it is available, or a portion of the transient galactose which has been diverted to furnish metabolic energy to the tissue.

With regard to the latter possibility, the ability to metabolize galactose has been well documented for intestinal tissue from two species of animals. Everted sacs of hamster intestine placed in galactose solutions convert some of the galactose to lactic acid[23], some to glucose, and some to CO_2 (see ref. 24). The conversion of galactose to CO_2 has also been demonstrated in slices of rat intestine[25]. In discussing these conversions, LANDAU AND WILSON[24] suggest that phosphorylation must be involved, with the implication that galactose is metabolized in intestine by the same pathways as in liver and erythrocytes[26]. The results reported here provide an experimental foundation for these suggestions.

In considering the results of the quantitative determinations, one may inquire whether the hexose 1-phosphates present in intestinal tissue during galactose absorption represent a significant proportion of the organic phosphates of the tissue. Since the present data applies to whole (small) intestine, and earlier workers have analyzed only the mucosa, comparison is difficult. However, if a tissue dry matter content of 20% is assumed, one may calculate that 19 μmoles of glucose 1-phosphate plus galactose 1-phosphate/100 g fresh tissue is equivalent to 29 mg hexose 1-phosphate P/100 g dry tissue. LASZT AND SÜLLMANN[6] quoted a value of 96 mg and LUNDSGAARD[7] a value of 346 mg for total acid-soluble organic P/100 g dry wt. in rat intestinal mucosa during glucose absorption.

The levels of intestinal galactose 1-phosphate achieved during galactose absorption were not significantly different in animals raised on stock, glucose-supplemented, and galactose-supplemented diets. Three animals of the lactose-fed group had below average levels of both hexose 1-phosphates during galactose absorption. However, the data are not extensive enough to warrant the conclusion that this is a typical effect of lactose feeding.

The finding that one of the fractions (peak 4, Fig. 1) from the pooled intestinal extracts consists largely of acid-stable phosphate, with galactose apparently the major sugar, suggests that galactose 6-phosphate may be present in intestinal tissue during the absorption of galactose. In view of the fact that two previous investigators[4,5] have obtained similar indications, this possibility should be further explored.

ACKNOWLEDGEMENT

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INFRARED SPECTRA OF CRYSTALLINE POLYSACCHARIDES

VI. EFFECT OF ORIENTATION ON THE TILTING SPECTRA OF CHITIN FILMS*

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SUMMARY

The tilting spectra of doubly oriented crab chitin crystallites and blowfly larval cuticle chitin were observed. It has been noted that in addition to the uniaxial orientation, these samples possess different selective uniplanar orientations. For the crab chitin crystallites the 100 crystallographic planes are parallel to the film surface while for the blowfly larval chitin the 001 planes are parallel to the film surface. Based on these orientations and the observed tilting effects, a number of previously made band-assignments were confirmed and new suggestions were made for interpreting the bands at 3106, 2962 and 1619 cm^{-1}.

INTRODUCTION

In the two previous reports of this series[1,2] the general principles for interpreting and the practical procedures for obtaining "tilted" infrared spectra of polymers were discussed as well as the general interpretation of the spectra of chitinous materials. The present study is an examination of the "tilting" effects which were observed in the infrared spectra of two chitin samples: crab chitin crystallites and larval chitin of blowfly. These two samples were chosen because they showed double orientation (*i.e.* one axis of the unit cell oriented in a single direction and one crystallographic plane parallel to the surface) which usually results in pronounced "tilting" effects.

From the tilting spectra of samples with well-defined crystallographic orientation it is frequently possible to obtain confirmation of band assignments which are made more or less empirically. Measurements such as described in the present study can also allow a choice to be made among a number of potential hydrogen bonding sites for a given hydroxyl[3]. Finally the importance of a full interpretation of the chitin spectrum cannot be overemphasized since it is a prototype of numerous biologically important polysaccharides which contain the aminoacetyl group.

EXPERIMENTAL

The preparation of rod-like crystallites from crab chitin has been described[2] previously. The clear transparent films which could be prepared in any thickness by drying down a dilute suspension of crystallites or by spreading unidirectionally a concentrated (birefringent gel) suspension[4] of the crystallites proved to be ideal for spectroscopic study.

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* Part V of this series: *J. Polymer Sci.*, 43 (1960) 85.

Purified blowfly larval chitin was kindly supplied by Dr. K. M. RUDALL of Leeds, England. The preparation and the morphological characteristics of this material have been reported[5]. The particular sample studied was 8–10 μ thick, somewhat more than desirable for infrared study. The sample had been stretched 30–40 % to give a moderate degree of uniaxial orientation.

Typical X-ray diagrams shown by the samples used in this investigation are shown in Fig. 1. The degree of uniaxial orientation was sufficient to show good infrared dichroism in the two cases[2]. One would conclude, however, from the similarity of the X-ray orientations and the higher dichroism of the crystallite sample that the blowfly chitin contained more noncrystalline material. This is reasonable in view of the fact that it has not undergone hydrolytic treatment.

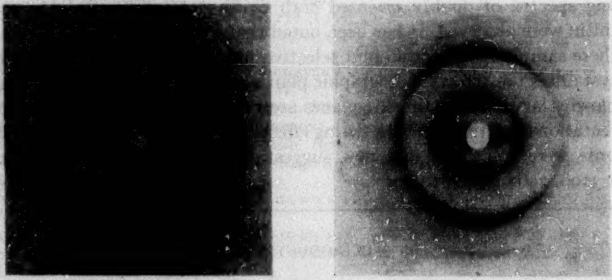


Fig. 1. X-ray diagrams of oriented films of crab chitin crystallites (left) and blowfly larval chitin (right). X-ray beam was parallel to the film surface and surface normal pointing to the equator.

The most significant feature of the X-ray diagrams is the orthogonal relation of the two paratropic reflections (the two most intense ones). For blowfly chitin the 001 (inner of the two) is equatorial but for the crab crystallite sample it is meridional, and *vice versa* for the outer 100 reflection. (The apparent diffuse equatorial maximum of the 001 reflection for the crab crystallites is due to "white" radiation.) This indicates that within the crystallite sample the 100 plane has preferred orientation parallel to the surface plane while the 001 is parallel to the surface of the blowfly chitin sample. These two planes correspond to the mutually perpendicular lateral faces of the chitin unit cell[6] (*cf.* Fig. 2). It should be noted that the double orientation of both samples is far from perfect.

The spectra were recorded in the 3 μ region by means of a Perkin-Elmer model 112 spectrometer using a LiF prism. For the 1800–650 cm^{-1} region a Perkin-Elmer model 21 spectrometer with NaCl optics was used. The settings for recording the tilting spectra have been described previously[1]. The spectrometer slit is used as a vertical reference axis for describing these settings so that the orientation of the fiber axis (f.a.) will be described below as perpendicular or parallel with reference to the slit. The electric vector is kept in a horizontal position, *i.e.* perpendicular to the slit. A tilting spectrum is obtained when the normal to the sample surface is at some angle θ

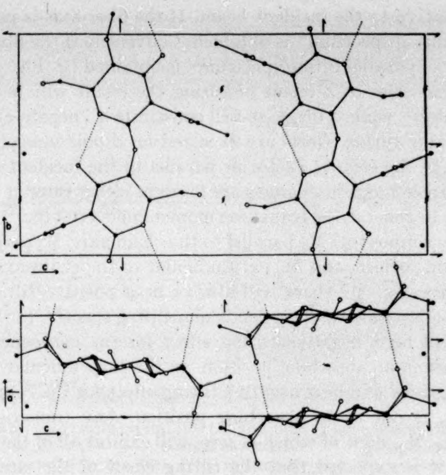


Fig. 2. Unit cell of chitin (after CARLSTRÖM[6]).

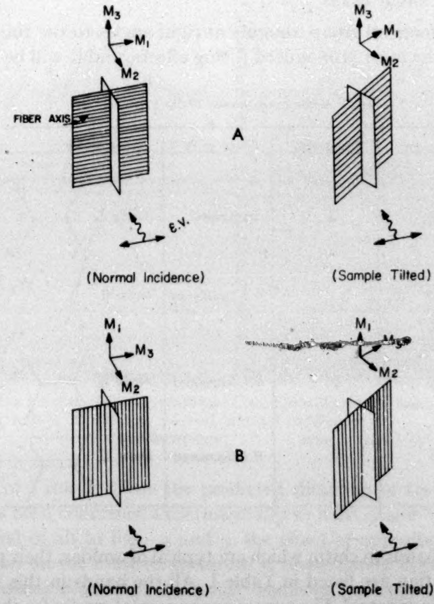

M3
M1
M2
FIBER AXIS
A
E.V.
(Normal Incidence)
(Sample Tilted)
M1
M3
M2
B
(Normal Incidence)
(Sample Tilted)

Fig. 3. Tilting effects of doubly oriented films showing settings for observing: A, parallel tilting spectrum; B, perpendicular tilting spectrum.

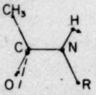
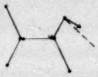
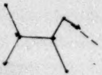
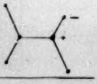
($0° < \theta < 70°$) relative to the incident beam. If the fiber axis is parallel to the slit, a "perpendicular tilting spectrum" is obtained. Conversely if the fiber axis is perpendicular to the slit, a "parallel tilting spectrum" is obtained (*cf.* Fig. 3). If a particular absorption band increases as a result of tilting the result will be referred to as a "positive tilting effect" while a decrease will constitute a "negative tilting effect".

The most striking tilting effects are expected for dipole moment changes which are either parallel to the electric vector or parallel to the incident beam for normal incidence. These dipole moment changes are brought either into, or out of absorbing position by tilting. In general, the transition moment of every vibrational mode can be considered to have components M_1 parallel to the chain axis, M_2 perpendicular to the doubly oriented film surface, and M_3 perpendicular to the chain axis and parallel to the film surface (see Fig. 3). There will always be a positive tilting effect for the component M_2 in both parallel and perpendicular tilting spectra. In the parallel tilting spectrum, there will be a negative tilting effect for the component M_1 while the component M_3 is not in an absorbing position. In the perpendicular tilting spectrum, on the other hand, there will be a negative tilting effect for the component M_3 while the component M_1 is not in an absorbing position. Any transition moment with components M_1, M_2, M_3, none of which is zero, will exhibit all of the foregoing tilting effects. However, it is expected that the tilting effect of the stronger component should be predominant.

Tilting effects for the amide bands

The planar aminoacetyl group, roughly at right angles to the 100 and 001 planes, is expected to show the most pronounced tilting effects, and it will be considered first.

Table I. Amide bands in chitin (700–1700 cm^{-1})

Freq. cm^{-1}	Polarization	Effect of D_2O	Interpretation
1652	$\perp$	Unchanged	Amide I 
1555	$\perp$	R.I. Decreased	Amide II 
1310	$\perp$	R.I. Decreased	Amide III 
730	$\parallel$	R.I. Decreased	Amide V 

The absorption bands in chitin which are typical of amides, their polarization and the effect of deuteration are listed in Table I. All the bands in this table have been identified in the chitin spectra[2]. The approximate normal modes for these amide bands are taken from the study of the spectrum of N-methyl acetamide[7]. The broken line in

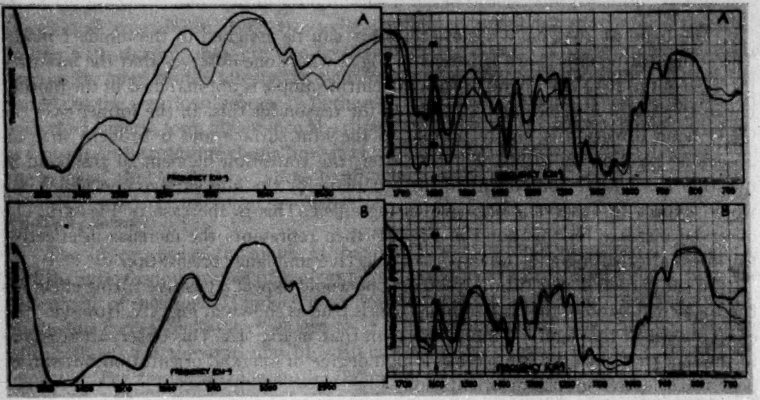


Fig. 4. Tilting spectra for crab chitin crystallites (A) and blowfly larval chitin (B) with electric vector and fiber axis both ⊥ to slit. (A) ———, normal incidence; ··········, sample tilted 60° (by rotating about a vertical axis parallel to the slit). Film thickness $\simeq 6\ \mu$. (B) ———, normal incidence; ··········, sample tilted 50°. Film thickness $\simeq 8\ \mu$.

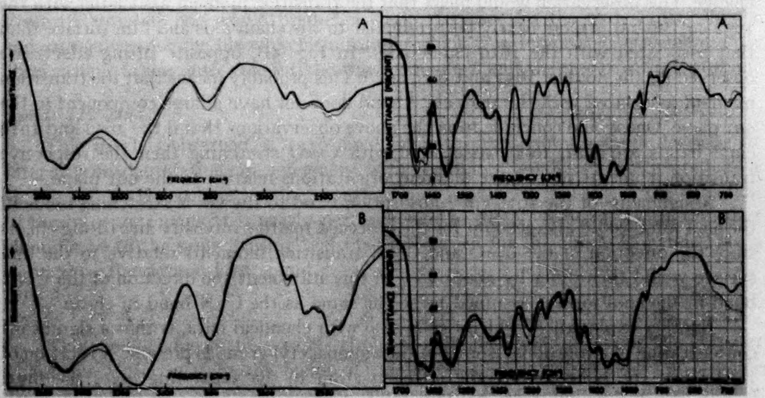


Fig. 5. Tilting spectra for crab chitin crystallites (A) and blowfly larval chitin (B) with electric vector // to slit and fiber axis ⊥ to slit. (A) ———, normal incidence; ··········, sample tilted 45°. (B) ———, normal incidence; ··········, sample tilted 50°.

the last column of Table I shows the predicted direction of the transition moment vector which has been confirmed experimentally in most cases[8,9].

Referring first of all to Figs. 4 and 5, the effect of opposite selective uniplanar orientation, *per se*, on the infrared spectrum is immediately apparent on comparing the relative intensities of the 1652–1619 cm^{-1} bands *vs*. the 1555 cm^{-1} band for normal incidence. The intensity ratios are reversed for the two samples reflecting their different preferred orientations.

Amide I band

The band at 1652 cm^{-1} almost certainly can be assigned to the amide I mode. The actual tilting effects are apparent in Fig. 4 where one observes that the intensity increase of the 1652 cm^{-1} band of the crab chitin sample is not matched in the blowfly chitin spectrum. A glance at Fig. 2 shows the reason for this. In the former case the C=O bond direction is nearly normal to the film surface and is brought into an absorbing position by tilting. For the latter, the transition moment of the bond in question has a large component in the 001 plane or the film surface and hence one would expect to observe a negative tilting effect. This is the case in Fig. 5B. The observed increase for this band in Fig. 4B then represents the increase in effective thickness after tilting and the imperfect selective uniplanar orientation.

In the perpendicular tilting spectrum one would expect a positive tilting effect for the 1652 cm^{-1} band of the crab sample. This is true as shown in Fig. 5A. However, the effect shown in this figure is much less than that in Fig. 4A. This observation is due to the fact that the crab sample has a high degree of uniaxial orientation but rather poor 100 selective uniplanar orientation.

The interpretation of the 1619 cm^{-1} band is not clear. The tilting effects of this band are noteworthy. The band shows a positive tilting effect in Fig. 4A and Fig. 5A. In Fig. 4A the intensity of this band is greater than that of the 1652 cm^{-1} band for normal incidence. When the sample is tilted, the relative intensities of the two bands change. This observation would mean that the transition moment associated with the 1652 cm^{-1} band is more nearly perpendicular to the chain axis and film surface than that associated with the 1619 cm^{-1} band. In Fig. 5B, opposite tilting effects are observed for the doublet 1652 and 1619 cm^{-1}. This probably means that the transition moment associated with the 1619 cm^{-1} band does not have a large component in the 001 plane. One would conclude from the above observations that if the 1652 and 1619 cm^{-1} bands are both to be associated with C=O stretching then the respective transition moments must have different orientations relative to the 001 plane.

Assignment[2] of the 1619 cm^{-1} absorption band to the C=N stretching mode of the enol form would also account for the observed relative intensity and tilting effects since the direction of the C=O and C=N transition moments relative to the film surface would then differ by about 40°. In this statement the direction of the C=N bond in the enol form is assumed to be the same as the C–N bond in chitin.

Another explanation, which ties in also with chemical data, is that a significant concentration of a rotational isomer of the aminoacetyl group is present. A rotation of 180° about the bond linking the nitrogen atom to the sugar ring gives the most acceptable conformation. The N–H bond direction remains the same but there is a significantly different C=O direction. The perpendicular dichroism of the amide I band would be unchanged but assignment of the 1619 cm^{-1} band to the amide I band of this rotation isomer could explain the difference in behavior of the two bands on tilting. This rotational isomer would necessarily represent some kind of disorder within the crystal. Accordingly, a density measurement was made on the crystalline N-acetylated chitoheptaose which shows the same X-ray diagram and infrared spectrum as native chitin. A value of 1.424 g/ml was obtained which is to be compared with the theoretical value of 1.462 (see ref. 6). The evidence then from the density measurement is not opposed to the hypothesis.

Chemists have frequently referred to the fact that chitin seems to have two kinds of acetyl groups[10]; one readily saponifiable but the other more resistant. If this is a question of accessibility then the foregoing hypothesis is all the more attractive. In line with this one may cite the fact[2] that the 3106 cm^{-1} N–H stretching band is apparently more readily affected by deuteration than the one at 3264 cm^{-1}. Within the bounds of the rotational isomer hypothesis, the former frequency could be associated with the isomeric form also. Since the direction of the N–H bond is changed by 180° as a result of the rotation, the dichroism of the associated band would be expected to be the same as that of the crystalline band, which is the case[2,5].

Amide II band

The appearance of the amide II′ band at 1470 cm^{-1} on deuteration of the chitin and the near disappearance of the 1555 cm^{-1} band leaves little doubt concerning the assignment of the amide II mode[2]. The direction of this transition moment is about 17° off the line corresponding to the pure N–H in-plane bending[7–9]. The observed positive tilting effects for the crab and blowfly chitin samples shown in Fig. 4 are not unexpected. The transition moment has components in a plane normal to the surface of the films which are brought into an absorbing position by tilting. (Note that the rather large tilting effect of this band in Fig. 4A but not in Fig. 5A is due to high uniaxial but poor selective uniplanar orientations.)

Because the transition moment does not lie directly in the plane of the film or normal to it, the setting used to obtain Fig. 5 is not favorable for tilting effects. From the unit cell one would predict a slight decrease for the crab chitin sample and an increase for the blowfly chitin sample which is in reasonable agreement with observation, taking into account the thickness effect and the imperfect orientation.

Amide III band

This band is observed at 1310 cm^{-1} in chitin and shows a large decrease in intensity on deuteration, in agreement with theoretical analysis[7], which predicts a significant N–H in-plane bending contribution. As shown in Table I the direction of the transition moment is nearly perpendicular to the N–H bond, a situation that should lead to the same "tilting" effects as observed for the amide II band. This is verified by the data in Figs. 4 and 5.

Amide V band

This mode is due entirely to N–H out-of-plane bending, *i.e.*, along the polymer chain axis. Unfortunately, this region of the spectrum is also rich in O–H out-of-plane bending modes which can cause some confusion in interpreting the tilting spectra. For the setting in Fig. 4 a "negative tilting" effect is expected for both samples. In fact, for the crab chitin sample a decrease is observed but the blowfly chitin sample shows an increase. In Fig. 5, similar tilting effects were observed for both samples. Most probably these anomalies are due to the positive contribution of a broad spectrum of O–H bending frequencies in the less-crystalline blowfly chitin. The O–H bands of the cellulose spectrum show positive tilting effects in this region[1].

NH stretching frequencies

Since the NH bond is nearly parallel to the C=O bond, the tilting effects of the NH stretching band should be similar to those of the amide I band. From the spectra shown in Figs. 4 and 5, it is interesting to note that the tilting effects of the bands at 3264 and 1652 cm^{-1} are very similar. The same is true for the bands at 3106 and 1619 cm^{-1}. These observations would probably support the assignment of both the 3264 and 3106 cm^{-1} bands to N–H stretching modes as discussed above.

Tilting effects for carbon-hydrogen modes

The setting used for Fig. 4A is ideal for observing tilting effects due to the C–H stretching modes which are nearly normal to the 100 plane. The positive tilting effects for the 2878 and 2890 cm^{-1} bands in Fig. 4A confirm their assignment to CH stretching modes. In Fig. 5A a similar intensity increase would be expected if the sample had a perfect selective uniplanar orientation. The very moderate positive tilting effect observed is certainly due to imperfect selective uniplanar but high uniaxial orientation for the crab chitin sample, as already mentioned in the discussion of amide bands.

The blowfly chitin sample shows only a thickness effect in Fig. 4B and a decrease in intensity in Fig. 5B, as expected for a sample with selective orientation of the 001 plane.

The only other carbon-hydrogen stretching frequency showing tilting effects is the 2962 cm^{-1} band which was assigned[2] to the CH_3 antisymmetric stretching mode. The tilting effects, although small, suggest a dipole moment change along the $C-CH_3$ bond which would be characteristic of the symmetric CH_3 stretching mode. The reason for this anomaly is not known. However this band could be assigned to the CH_3 symmetric stretching mode by assuming that the CH_3 antisymmetric stretching mode absorbs at a higher frequency but with very weak intensity in the infrared.

The CH_3 symmetric bending mode assigned to the 1378 cm^{-1} band shows the tilting behavior which is expected from the molecular model. It should be remembered, however, that this band may, in fact, be composite since the C–H bending frequency could occur here also.

Tilting effects offer little help in deciding which of the 1430 and 1420 cm^{-1} bands belongs to the CH_2 symmetric bending mode, since tilting effects were found only in one of the settings. All that can be said is that observations are not contrary to the conformation proposed previously[2] for the hydroxymethyl group.

Tilting effects for hydroxyl modes

Since the hydroxyl stretching frequencies were assigned to bands showing parallel polarizations these bands should show a negative tilting effect for the setting in Fig. 4, as they actually do.

Interpretation of tilting effects for the O–H in-plane and out-of-plane bending modes is less straight-forward because band assignment to the individual hydroxyls has not been made. However, if the conformation of the hydroxymethyl group suggested previously[2] be correct, then the 685 cm^{-1} band is most probably associated with its out-of-plane bending mode. In this case, the transition moment would be oriented at an angle of about 45° to the chain axis and in the 100 plane. This orientation could explain the opposite "tilting" effects observed for the two samples in Figs. 4 and 5.

DISCUSSION

Although imperfect orientation did not always allow the full effect of the orthogonal uniplanar orientations to be observed in the tilted spectra of these chitin samples, major differences nevertheless were found. These differences were in accord with predictions based on the X-ray unit cell recently proposed by CARLSTRÖM[6]. Since this structure allows for complete N-H···O=C hydrogen bonding the assignment of the 3445 cm^{-1} band to a free N-H group[10] is less acceptable than its assignment to the O-H stretching mode, which has been proposed[2]. However, the apparent presence of two NH stretching and two C=O stretching bands is still not explained satisfactorily. It is noteworthy that the frequency difference between the 1652 and 1619 cm^{-1} bands is about the same as the frequency shift in the $\alpha \rightarrow \beta$ transformation of polypeptides[8,9].

The finding of two chitin samples with different selective planar orientation has its analogue in the cellulose field where samples with 101 and $10\bar{1}$ planar orientation are available[1]. The value of such samples in helping to associate infrared absorption bands with the proper chemical group is clear. From a biophysical point of view it is more important to understand the relation between crystallite shape and planar orientation. Planes rich in hydrogen bonds (001 for chitin) are usually expected to show more rapid growth. Drying stresses may then be expected to orient these larger planes parallel to the membrane surface. Since low angle X-ray diffraction[11] seems to confirm that the widest face of the crystallite is parallel to the film surface the existence of films with the same unit cell but orthogonal planar orientation suggests that factors other than hydrogen bond direction control the crystallite shape.

When hydrolysis is involved, as in the present case, for the preparation of crystallites, the effect of charged groups (such as NH_3^+) in causing cleavage other than that expected crystallographically should not be overlooked. Thus, addition of a proton to the few amino groups which are known to be present in chitin, could bring about interactions which would readily cause cleavage along the 100 plane.

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ON THE NATURE AND MODE OF ACTION OF THE COPPERPROTEIN, TYROSINASE

I. EXCHANGE EXPERIMENTS WITH RADIOACTIVE COPPER AND THE RESTING ENZYME

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SUMMARY

1. Radioactive ^{64}Cu and ion-exchange techniques have been applied to study the incorporation of radioactive copper into resting tyrosinase.

2. Under the experimental conditions, the radiocopper incorporation into the enzyme was found to be small and decreased with increasing purification of the enzyme.

3. The data are used to present the view that the functional copper of the enzyme is bonded to the protein in a non-dissociable bond.

4. The results are explained in terms of two types of copper sites (active and non-exchangeable; inactive and exchangeable) in the impure and/or partially inactivated enzyme.

INTRODUCTION

The enzyme tyrosinase, also called polyphenol oxidase[1,2], has long been known to contain copper as a factor essential for its activity[3-5]. However, very little is known as to the nature of the bond between the copper and the protein. The mode of function of the tyrosinase copper during its catalysis of the aerobic oxidation of mono- and *o*-dihydric phenols is not understood.

A number of years ago, JOSELOW AND DAWSON[6,7] used ^{64}Cu and ion-exchange techniques in an investigation of the nature of the copper–protein bond in the enzyme ascorbic acid oxidase. They found that the copper–protein bond in the resting enzyme is non-dissociable, as evidenced by the fact the non-functioning enzyme did not exchange its copper when exposed to radioactive cupric ions. The approach used in those studies has provided methods for obtaining valuable information about the properties of other copper proteins.

Tyrosinase is particularly interesting because it is a copper protein that possesses two essentially different catalytic activities, *i.e.*, monophenolase and *o*-dihydric-

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[*] This paper represents part of the work submitted by H. DRESSLER to Columbia University in partial fulfillment of the requirements for the Ph.D. degree in Chemistry.

phenolase activity[1]. It plays an important role in the pigmentation processes of many plants and animals and is believed to be involved in the respiration of some plant species[8,9]. The present investigation deals with exchange experiments between radioactive cupric ions and resting (non-functioning) tyrosinase.

EXPERIMENTAL

Tyrosinase

The common mushroom, *Psalliota compestris*, served as the enzyme source. The method of preparation used was that described earlier by workers in these laboratories[10]. The catecholase activity of the enzyme was measured by the chronometric method[11]. The cresolase activity was measured manometrically[12] and the protein content of the enzyme preparations was estimated by determination of the dry weights of dialyzed aliquots[10]. The characteristics of the enzymes used in this investigation are summarized in Table I.

TABLE I

CHARACTERISTICS OF THE TYROSINASE PREPARATIONS USED IN THE EXCHANGE EXPERIMENTS

Enzyme preparation No.	*mg/ml dry wt.*	*μg Cu/ml*	*% Cu*	*Catecholase activity per*			*Cresolase activity per*			*Catecholase cresolase activity ratio*
				ml	*mg*	*μg Cu*	*ml*	*mg*	*μg Cu*	
C308	4.9	7.0	0.14	11700	2390	1670	315	64	45	37.2
C305D2	4.4	7.4	0.17	10350	2450	1400	285	64	37	36.4
C312-2	2.2	4.5	0.20	5730	2600	1280	118	53	26	48.6
C305D	4.4	7.4	0.17	12100	2730	1640	280	64	38	43.2
C313-1	4.8	7.1	0.15	13100	2740	1850	165	34	23	79.3
C311-2	5.9	8.8	0.15	19100	3240	2170	365	62	42	52.3
C314-M	7.2	11.7	0.16	25100	3500	2140	510	71	44	49.2
C306D	5.0	4.4	0.09	3640	728	830	348	70	79	10.5
C307D	6.3	7.3	0.12	10100	1600	1390	935	148	128	10.8

In general, two types of tyrosinase preparations can be distinguished[13]. Those of lower molecular weight having a ratio of catecholase to cresolase activity of about 20 or higher are called "high catecholase" enzymes. Those having a higher molecular weight and a catecholase to cresolase activity ratio of about 10 or lower are called "high cresolase" enzymes. Intermediate preparations can also be obtained. In Table I the seven "high catecholase" and the two "high cresolase" preparations are arranged in the order of increasing catecholase activity per milligram of dry weight (Column 6) which is a good measure of the purity of the preparation.

Measurement of copper

The method of WARBURG AND KREBS[14] was used to estimate the copper content of the several enzymes employed, of the cupric salt solutions used and of the ion-exchange column effluents. The method was satisfactory for the enzyme and standard copper solution analysis, detecting as little as 0.01 μg of copper with a precision of ± 5 %. However, the analysis of the column effleunts was less satisfactory (precision estimated to be ± 8 %). Several attempts to apply colorimetric methods[15,16] which were found to give good results with standard copper solutions, were unsuccessful in the estimation of the trace amounts of copper in the effluents.

Ion exchange resin

Analytical grade Amberlite IR-100*, a cation exchanger, was used in columns of the type described by JOSELOW AND DAWSON[6]. These columns contained about 3 g of resin (air dried) which had a capacity for copper far in excess of the actual needs of the experiment. The resin was conditioned and used in the Na^+ form as previously described[6]. The removal of cations by these columns depends on the rate of flow. It was established that a rate of flow through the resin column of about one drop per 40 sec (corresponding to 10 ml of effluent collected in 70 to 100 min) guaranteed the complete removal of much larger amounts of cupric ions than used in the exchange experiments. The enzyme was not deactivated or retained by the resin. When enzyme solutions were passed through the Amberlite IR-100 column, all of the tyrosinase activity and copper was recovered in the effluent. It was also found that the Amberlite resin removed added cupric ions quantitatively from acetate buffer solutions (pH 5.7) containing enzyme. The pH 5.7 was selected because it is above the isoelectric point of tyrosinase and in the range of optimum enzyme activity.

Radioactive copper solutions

The Brookhaven National Laboratories of Long Island, N.Y., supplied the ^{64}Cu (half-life 12.8 h) in wire form. The wire was dissolved in hot dilute nitric acid and dilutions of the resulting stock solution to the desired copper concentration were made with 0.1 *M* acetate buffer of pH 5.7. The short half-life of this isotope made necessary the completion of each experiment within a relatively short time. The radioactivity of the ^{64}Cu did not impair the catalytic activities of tyrosinase**.

The counting procedure

The manipulations were similar to the previously described operation[7]. Aliquots of the radioactive solutions were pipetted into the center well of aluminum planchets designed to fit a flow-type (Q-gas) counter. The samples were evaporated under an infrared lamp, then allowed to cool in a desiccator over phosphorus pentoxide before counting. Duplicate specimens were prepared and counted for all samples. All counts were corrected for decay and background. In none of the experiments was the counting rate high enough to necessitate coincidence corrections.

Actual exchange experiments

In each experiment a given amount of tyrosinase was mixed with an amount of radioactive cupric ions equivalent to the amount of enzyme copper. These solutions, buffered to pH 5.7 with 0.1 *M* acetate buffer, were let stand at room temperature (24–26°) for a certain length of time with frequent shaking and then they were passed through the cation exchange columns. The column effluents were subsequently assayed for radioactivity, enzyme activities, and copper content. The radioactive comparison standard solutions were identical with the mixtures investigated but were not passed through the Amberlite columns. As a measure of the exchange taking place, the counting rate of the effluents was expressed as a percentage of the counting rate of the comparison standard. This percentage is reported as the percent radioactivity incorporated into the enzyme. It should be kept in mind that, in the case where an equivalent amount of radioactive copper and enzyme are involved and no adsorption of cupric ions to the protein occurs, the percentage radioactivity incorporated into the enzyme can never exceed 50 %, *i.e.*, one half the radioactivity of the comparison standard. In this case of maximum exchange there has been a complete random distribution of the total added radioactivity between the enzyme copper and the ionic copper in the solution.

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* Rohm and Haas Co., Philadelphia, Pa. (U.S.A.).

** FRIEDEN[17] has previously reported that added cupric ions do not affect the activity of tyrosinase.

Other details

The solutions of reagents and buffer salts were prepared with distilled water that had been redistilled using an all Pyrex glass still with a hot wire around the reflux condenser to break the film. Such "copper-free" water contained less than 0.01 μg of copper /ml. The buffer salts were twice crystallized from copper-free water. Solutions of ascorbic acid (Chas. Pfizer Company) were freshly prepared before each experiment in which they were involved. Catechol and *p*-cresol (Eastman-Kodak Company) were resublimed and redistilled, respectively, prior to use.

RESULTS

The exchange experiments between resting tyrosinase and radioactive cupric ions at 40–50 min exposure time are summarized in Table II. The degree of purity of the enzymes, expressed in specific activities, is listed in column 2 of this table. The copper content of the cation exchange columns corresponded, within experimental error, to the copper content of the enzyme (columns 3 and 4). Small amounts of radioactive copper were incorporated into the enzyme (column 7). The data in Table II have been arranged in the order of decreasing exchange so as to indicate the inverse relationship that was found to exist between the specific activity ("purity") and the extent of radiocopper incorporation. This inverse relationship appears to be essentially linear

TABLE II

PERCENT RADIOACTIVITY INCORPORATED INTO THE RESTING ENZYME AFTER 40–50 MIN OF CONTACT WITH RADIOCOPPER

The amount of ^{64}Cu involved was the same as the copper content of the enzyme. Amberlite IR-100 column used.

Enzyme preparation No.	*Catecholase activity per mg dry wt.*	*Cu in enzyme before μg*	*Cu in effluent after μg*	*% in effluent of the original*		*% radioactivity* incorporated into the enzyme*
				Catecholase activity	*Cresolase activity*	
C306D	728	2.2	2.0	—	—	9.7 ± 0.8
C307D	1600	3.7	3.7	67	81	7.1 ± 1.3
C305D2	2450	3.7	3.4	69	57	5.0 ± 0.6
C305D	2730	3.7	3.3	73	73	3.6 ± 0.3
C308	2390	3.5	4.0	103	—	3.4 ± 0.4
C312-2	2600	4.5	4.1	106	61	1.9 ± 1.3
C313-1	2740	3.6	3.9	97	66	1.9 ± 0.7
C314M	3500	4.7	4.7	81	—	1.9 ± 0.3
C311-2	3240	4.4	3.9	67	48	1.1 ± 0.1

* The error analysis of the radioactive assay was carried out according to G. FRIEDLANDER AND J. W. KENNEDY, in *Introduction to Radiochemistry*, J. Wiley, New York, 1949.

when the % radioactivity incorporated into the enzyme is plotted against the catecholase activity/mg dry wt. Extrapolation of the linear relationship in the direction of higher enzyme activity indicates that an enzyme preparation having a specific activity of about 4000 catecholase units/mg dry wt. would not undergo exchange with radioactive cupric ions. This value of specific activity was that found by MALLETTE AND DAWSON[13] for homogeneous ("pure") tyrosinase. Unfortunately, an enzyme of comparable purity was not available for this investigation.

In another series of experiments, the dependence of the amount of radioactive copper incorporated into the enzyme on the length of contact time between the radiocupric ions and tyrosinase was established. The results of these experiments are presented in Table III. When these data are plotted, % incorporation *vs.* time, they indicate that a limiting value of exchange is approached after a period of time which appears to differ for each enzyme preparation. The limiting value again shows the inverse relationship between radioactive copper incorporation and the purity of the preparation, if the relatively high values for the highly purified preparation C 314 M are ascribed to a temperature effect.

TABLE III

PERCENT RADIOACTIVITY INCORPORATED INTO THE RESTING ENZYME ON VARYING THE TIME OF CONTACT WITH RADIOCOPPER

The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Enzyme preparation No.	*Catecholase activity per mg dry wt.*	*Time of contact with ^{64}Cu (min)*	*Cu in enzyme before µg*	*Cu in effluent after µg*	*% in effluent of the original*		*% radioactivity* incorporated into the enzyme*
					Catecholase activity	*Cresolase activity*	
C306D	728	40	2.2	—	—	—	9.7 ± 0.8
C306D	728	270	2.2	2.0	—	68	14.3 ± 0.9
C308	2390	45	3.5	4.0	103	—	3.4 ± 0.4
C308	2390	165	3.5	3.6	—	—	5.0 ± 0.5
C308	2390	285	3.5	3.7	83	—	6.7 ± 0.5
C308	2390	480	3.5	4.1	—	—	6.6 ± 0.5
C308	2390	1350	3.5	3.3	81	—	6.8 ± 0.5
C305D	2730	40	3.7	3.3	73	73	3.6 ± 0.3
C305D	2730	260	3.7	3.4	57	62	5.7 ± 0.6
C305D	2730	840	3.7	3.2	—	—	10.0 ± 0.1
C311-2	3240	45	4.4	3.9	67	48	1.1 ± 0.1
C311-2	3240	100	4.4	—	76	59	1.9 ± 0.2
C311-2	3240	180	4.4	3.9	—	—	2.4 ± 0.7
C311-2	3240	300	4.4	3.9	67	56	2.3 ± 0.7
C311-2	3240	480	4.4	4.2	—	—	2.8 ± 0.8
C311-2	3240	1170	4.4	4.1	74	59	5.0 ± 0.4
C314 M**	3500	45	4.7	4.7	81	—	1.9 ± 0.3
C314 M	3500	180	4.7	4.8	—	—	3.1 ± 0.4
C314 M	3500	360	4.7	4.9	58	78	4.6 ± 0.5
C314 M	3500	540	4.7	4.5	—	—	5.5 ± 0.4
C314 M	3500	1200	4.7	—	69	68	7.3 ± 0.4

* See footnote under Table II.

** All experiments with Prep. No. C314 M were carried out at 32° rather than 24–26° as with all other preparations.

DISCUSSION

Purified tyrosinase preparations do not lose their copper on dialysis against copper-free water under physiological conditions[2]. The cation exchange resin Amberlite IR-100 did not remove copper from the enzyme (Table II). These facts indicate that the copper is firmly bound to the tyrosinase protein. The low incorporation of radioactivity into highly purified tyrosinase (and the inferred failure of homogeneous tyrosinase to exchange copper) are further indication of the firm bond of copper to the tyrosinase protein. Indeed, these findings are evidence that the copper involved in the enzyme function is non-dissociable from the protein.

Previously, it has been shown that samples of resting ascorbic acid oxidase of varying degrees of purification did not exchange their copper with radioactive cupric ions[7]. Thus, tyrosinase behaved differently, since some incorporation of radioactivity was found. An inverse relation between the extent of exchange and the "purity" of tyrosinase was apparent. The enzyme lost activity during most experiments (Table II, columns 4 and 5), presumably due to the diluting of the enzyme stock solution and due to bringing it from refrigerator (storage) to room temperature. However, there does not appear to be any simple relationship between the enzyme inactivation and the amount of radiocopper incorporated into the preparation. Thus, as can be seen in Table II, the losses in catecholase and cresolase activities of enzymes C305 D2 and C 311-2 were quite similar but the amounts of radioactive copper incorporated were significantly different.

The observed incorporation of small amounts of radioactivity into tyrosinase might be explained on the basis of earlier observations and interpretations of KUBOWITZ[3]. This pioneer worker in the field of copper containing enzymes found that 100% reactivation of apo-enzyme (*i.e.*, enzyme from which the copper atoms had been removed with cyanide) required much more copper than was originally bound to the protein. He could not remove this excess copper by dialysis against water and concluded that copper could occupy inactive positions ("falsche Stellen") on the enzyme. Consequently it seems likely that there may be two types of copper-bonding sites in tyrosinase; active and inactive sites. If one assumes that the copper bound to the active sites is the more strongly bonded, it is to be expected that the purification of the enzyme will preserve these active sites preferentially. Thus, with increasing purification (higher specific activities), the number of more weakly bonded copper atoms decreases. It may be that these inactive copper sites are the only ones which undergo exchange with radioactive cupric ions. In other words the data obtained in this investigation suggests that resting tyrosinase undergoes exchange with radioactive cupric ions only when the enzyme contains copper bonded at inactive sites, *i.e.*, only when the enzyme is impure and/or partially inactivated.

ACKNOWLEDGEMENT

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ON THE NATURE AND MODE OF ACTION OF THE COPPERPROTEIN, TYROSINASE

II. EXCHANGE EXPERIMENTS WITH RADIOACTIVE COPPER AND THE FUNCTIONING ENZYME

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SUMMARY

1. The exchange reaction between functioning tyrosinase and radiocupric ions has been studied.

2. In general, high catecholase enzymes incorporated much more radioactive copper during catalysis of the oxidation of *o*-dihydric phenols than of monophenols.

3. High cresolase enzymes retained little radioactivity in all experiments.

4. The copper exchange data support the suggestion of two distinct activity sites in tyrosinase; *i.e.* catecholase and cresolase activity centers. It appears that the copper at cresolase activity sites is non-exchangeable. The magnitude of exchange of the copper atoms at catecholase activity sites seems to depend on the number of *o*-dihydric phenol molecules oxidized.

5. The catecholase sites of tyrosinase appear to be little or not at all involved in the oxidation of monophenols.

6. On the basis of the exchange experiments it is suggested that the oxidation of a monophenol by tyrosinase may not proceed via an *o*-dihydric phenol.

INTRODUCTION

In the previous paper[1], exchange experiments involving radioactive ^{64}Cu and resting (non-functioning) tyrosinase were described. Copper, not protein bound, was removed from these systems by means of ion exchange resins. It was found that the small amount of radio-copper incorporated into the enzyme, in the absence of substrate, decreased as the purity of the enzyme increased. These results were explainable in terms of two types of copper sites (active and non-exchangeable; inactive and exchangeable) in the impure and/or partially inactivated enzyme.

The present paper describes the results of similar exchange experiments involving radioactive ^{64}Cu and the copper of the catalytically functioning enzyme.

The substrates commonly used in studies of tyrosinase are *p*-cresol and catechol.

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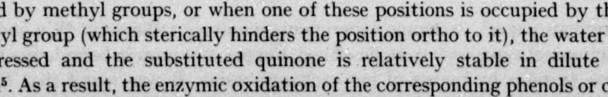
* This paper represents part of the work submitted by H. DRESSLER to Columbia University in partial fulfillment of the requirements for the Ph.D. degree in Chemistry.

Preliminary experiments showed that the cation exchange resin Amberlite IR-100 quantitatively removed ionic copper from acetate buffered aqueous solutions of these compounds. However, their aerobic oxidation as catalyzed by tyrosinase produces insoluble melanin-like pigments. These precipitates rapidly clogged the resin columns to such an extent that further passage of solution through the columns was impossible. This difficulty was overcome by employing 4-*tert.*-butylphenol, 3,4-dimethylphenol, and the corresponding substituted catechols as the substrates in this investigation. The reasons why these substrates were most satisfactory are given below.

The tyrosinase catalyzed oxidation of both mono- and *o*-dihydric phenols involves the formation of the corresponding *o*-benzoquinones. The stability of these *o*-quinones in the aqueous media is markedly dependent upon their structure. Furthermore, the amount of oxygen consumed and the amount of precipitate formed are dependent on the stability of the quinone. The complete enzymic oxidation of *p*-cresol (presumably *via* the corresponding catechol and thence 4-methyl-benzoquinone-1,2, which is unstable) requires 3 atoms of oxygen/mole of *p*-cresol under optimum conditions[2]. The oxidation of catechol via the unstable *o*-benzoquinone requires 2 atoms of oxygen/mole of substrate[2]. However, as can be seen in Table I, the enzymic oxidation of 4-*tert.*-butylphenol, 3,4-dimethyl phenol, and the corresponding catechols, requires 1 atom less of oxygen than the oxidation of *p*-cresol and catechol. The oxidation of each of these phenols and catechols resulted in a stable yellow solution (stable quinone) and no precipitate was produced.

The instability of *o*-benzoquinone in water, which corresponds to its ability to produce insoluble pigments, has been explained in terms of a reaction between the quinone and water to produce hydroxyhydroquinone as a transitory intermediate in the pigmentation reaction[2].



The hydroxyhydroquinone is not detectable in the system because it is very rapidly oxidized by another molecule of the *o*-benzoquinone to produce catechol and the unstable hydroxyquinone. When the 4,5 positions of the original *o*-benzoquinone are occupied by methyl groups, or when one of these positions is occupied by the bulky *tert.*-butyl group (which sterically hinders the position ortho to it), the water reaction is suppressed and the substituted quinone is relatively stable in dilute aqueous solution[5]. As a result, the enzymic oxidation of the corresponding phenols or catechols results in one less atom of oxygen uptake (see Table I) and no production of melanin-like precipitate*. In this connection the observation of CUSHING[6] that 4-*n*-butylphenol consumes 3 atoms of oxygen/mole on enzymic oxidation is of interest. It points up the fact that the *n*-butyl group does not have the steric effect of the *tert.*-butyl group.

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* In his review, *The Structure of Melanins*, MASON[3] has argued against the water reaction in favor of a direct polymerization of *o*-benzoquinones. His arguments, however, are not convincing, primarily because they do not account for the fact that *o*-benzoquinone is relatively stable in anhydrous media. When water is added to an anhydrous ether solution of *o*-benzoquinone (previously stable for a considerable period) the quinone rapidly disappears and the typical catechol melanin-like precipitate forms. This fundamental role of water in the *o*-quinone instability is readily followed spectrophotometrically[4].

In addition to the 4-*tert.*-butyl- and 3,4-dimethylphenols and catechols, the dipeptide L-tyrosyl-L-alanine was found to be a satisfactory substrate for tyrosinase in the exchange experiments. While tyrosine is sparingly water soluble, absorbs 4 atoms of oxygen and forms an insoluble melanin on enzymic oxidation, L-tyrosyl-L-alanine is water soluble, also absorbs 4 atoms of oxygen during complete enzymic oxidation, but gives a dark purplish colored solution (no precipitate).

TABLE I

TOTAL OXYGEN UPTAKE OF THE SUBSTRATES USED IN THE TYROSINASE COPPER EXCHANGE EXPERIMENTS

The oxygen absorption was determined in Warburg respirometers using flasks of 30 ml capacity; shaking rate was 120 oscillations/min; Temperature, 25°, pH 5.7. Total reaction volume was 5.0 ml, consisting of 2.0 ml of 0.1 M acetate buffer, 1.0 ml (5 mg) of gelatine solution, 1.0 ml (1 mg) of aqueous substrate solution, 0.5 ml of high catecholase enzyme (containing a minimum of 60 cresolase units *i.e.* a large excess of enzyme), and 0.5 ml of distilled water. In the case of 4-*tert.*-butylphenol, which is only slightly soluble in water, the system contained 10% ethanol.

Substrate	*Total O_2 consumed per 1 mg (cmm)*	*Total O_2 uptake in atoms per mol of substrate*
4-*tert.*-butyl phenol	148	1.99
3,4-dimethyl phenol	174	1.91
4-*tert.*-butyl catechol	64	0.96
4,5-dimethyl catechol	77	0.95

On passing 10 mg of 4-*tert.*-butylbenzoquinone -1,2 and 50 μg of cupric ions through an Amberlite column, a colorless effluent resulted in which no copper could be detected. In other words, the quinone apparently remained on the resin column but did not interfere with the removal of cupric ions by ion exchange.

EXPERIMENTAL

Tyrosinase

The various enzyme samples were obtained from the common mushroom, *Psalliota campestris*. The methods of preparation, estimation of copper content, protein content, and enzymic activities have been described elsewhere[1].

The exchange experiments

The preparation and use of the radioactive ^{64}Cu solutions, the counting procedure, and the use of Amberlite IR-100 as a resin for removing cupric ion from the enzyme have been described previously[1]. The exchange experiments were set up and run in the same manner as the resting enzyme experiments[1] except that various amounts of substrate were added to the reaction mixture at zero time. In each experiment the substrate was completely oxidized as established by manometric and spectrophotometric measurements[5]. All functioning enzyme exchange data were corrected by subtracting from the actual measurements the percent radio-copper incorporation of the resting enzyme exposed to ^{64}Cu for the corresponding time interval[1].

Materials

Catechol, *p*-cresol, and ascorbic acid solutions were prepared and used as described previously[1]. The 4-*tert*.-butylphenol (Distillation Products Industries) and the 3,4-dimethylphenol (Kahlbaum A.G.) were re-crystallized from copper-free water. Their melting points (99–100° and 62–63°, respectively) checked with the published records[7,8]. The 4-*tert*.-butylcatechol and 4,5-dimethylcatechol had been prepared earlier in these laboratories by ROTH[9]. A sample of L-tyrosyl-L-alanine ($\alpha_D^{23} = +7.84°$) was kindly supplied by Drd. E. F. ERLANGER AND D. M. KIRSCHENBAUM of the Department of Biochemistry, Columbia University. The synthesis of 4-*tert*.-butylbenzoquinone-1,2 is described elsewhere[5].

RESULTS

*Exchange of enzyme copper during the oxidation of 4-*tert.*-butylcatechol*

The incorporation of radioactive ^{64}Cu into the enzyme was found to depend on the amount of substrate employed and on the type of tyrosinase preparation used (Table II and Fig. 1). The maximum amount of substrate which could be used was 4 mg/5 ml total volume. At higher concentrations the solutions become turbid within a short time, indicating the beginning of precipitate formation and decreased stability of the 4-*tert*.-butyl-benzoquinone -1,2 (see ref. 5).

TABLE II

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON VARYING AMOUNTS OF 4-*tert*.-BUTYL CATECHOL

Reaction time, 50 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Type of enzyme	*Enzyme preparation No.*	*Cu in enzyme µg*	*Cu in effluent µg*	*mg of substrate*	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
					catecholase activity	*cresolase activity*	
High catecholase	C305D2	3.7	3.1	0.01	—	—	0
	C305D2	3.7	3.4	0.1	86	79	0
	C305D2	3.7	3.1	0.5	—	—	11.5 ± 1.2
	C305D2	3.7	3.2	1.0	—	—	28.1 ± 1.7
	C305D2	3.7	3.2	3.0	89	74	27.5 ± 1.7
High cresolase	C307D	3.7	3.7	0.2	—	—	0
	C307D	3.7	3.4	0.6	—	—	3.7 ± 1.5
	C307D	3.7	4.2	1.0	—	—	5.8 ± 1.7
	C307D	3.7	4.0	4.0	64	—	15.4 ± 1.6

Table II and Fig. 1 show that at any given substrate concentration the high catecholase preparation showed a higher copper exchange than the high cresolase preparation.

Although the radiocopper incorporation recorded with the high catecholase preparation at both 1.0 mg and 3.0 mg of substrate is about 28 %, indications were found that a figure close to the theoretical maximum of radioactivity in the enzyme, *i.e.* 50 %, would have been attained had it been possible to go to higher substrate concentrations. Namely, an indirect but considerable extension of the amount of substrate was achieved by oxidizing 1.0 mg of 4-*tert*.-butylcatechol with tyrosinase in the presence of 12 mg of ascorbic acid under the conditions of the exchange experiments. During this reaction the enzyme incorporated 45 % of the radioactivity of the comparison standard. Since tyrosinase is a very poor catalyst for the oxidation of ascorbic acid[*], one can expect that the 4-*tert*.-butyl-benzoquinone -1,2 formed was continuously reduced to 4-*tert*.-butylcatechol by the ascorbic acid until all the ascorbic acid had been consumed by oxidation to dehydroascorbic acid.

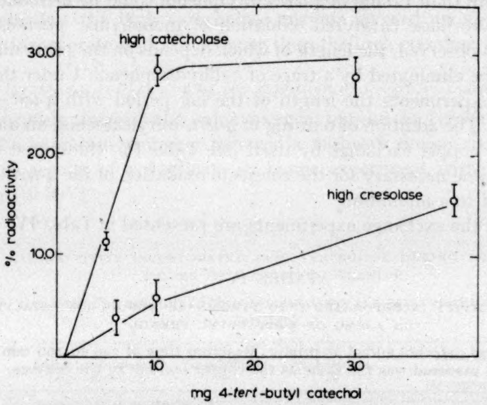

Fig. 1. Exchange with 4-*tert*.-butyl catechol as the enzyme substrate. Dependance of the exchange on the substrate concentration and enzyme type.

Exchange of enzyme copper during the oxidation of 4,5-dimethylcatechol

The findings are summarized in Table III. Only a high cresolase enzyme preparation was tested. The results were comparable to those obtained with a similar enzyme using 4-*tert*.-butylcatechol as the substrate.

TABLE III

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON VARIOUS AMOUNTS OF 4,5-DIMETHYL CATECHOL

Reaction time, 50 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

High catecholase enzyme preparation No.	*Cu in enzyme µg*	*Cu in effluent µg*	*mg of substrate*	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
				catecholase activity	*cresolase activity*	
C312.2	4.5	4.7	1.0	106	54	28.4 ± 2.0
C312.2	4.5	4.3	1.0	—	—	30.8 ± 1.9
C312.2	4.5	4.6	2.0	—	—	34.7 ± 3.2

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[*] The large amount of tyrosinase which had to be used in each of the exchange experiments was found to catalyze the oxidation of ascorbic acid very slowly. This rate of oxidation was comparable to the rate of oxidation of ascorbic acid catalyzed by a similar amount of gelatine and cupric ions. The latter was determined by Dr. R. J. Magee and Miss N. Benhamou in these laboratories.

*Exchange of enzyme copper during the oxidation of 4-*tert.*-butyl-phenol*

Because of the very limited solubility of 4-*tert.*-butylphenol in water, this substrate was used in the form of 1 ml of an aqueous solution containing 10% ethanol and 1.0 mg of the phenol. A preliminary study of control systems established that the small amount of alcohol thus introduced into the system did not inactivate the enzyme or alter the exchange of the resting enzyme. Due to the substrate solubility difficulties not more than 1.0 mg of 4-*tert.*-butylphenol could be introduced.

Before the tyrosinase catalyzed oxidation of monohydric phenols gets under way a lag period is observed, the length of which depends on the particular substrate. The lag time can be eliminated by a trace of *o*-dihydricphenol. Under the conditions of the exchange experiments the length of the lag period with 4-*tert.*-butylphenol was about 25 min. The addition of 0.01 mg of 4-*tert.*-butylcatechol, an amount which had not produced copper exchange by itself (see Table II), eliminated the lag time completely[5]. The time necessary for the complete oxidation of the 4-*tert.*-butylphenol could thus be kept to a minimum.

The results of the exchange experiments are presented in Table IV.

TABLE IV

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON 1.0 MG OF 4-*tert.*-BUTYL PHENOL

0.01 mg of 4-*tert.*-butyl catechol added as primer. Reaction time of 240 to 260 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Type of enzyme	*Enzyme preparation No.*	*Cu in enzyme µg*	*Cu in effluent µg*	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
				catecholase activity	*cresolase activity*	
High catecholase	C304D2	3.5	3.1	49	39	2.7 ± 1.3
	C304D2	3.5	3.2	—	—	4.2 ± 1.3
	C304D2	3.5	3.0	—	27	0.5 ± 1.8
	C304D2	3.5	3.2	—	—	0.5 ± 1.5
	C305D	3.7	3.3	53	57	0.8 ± 0.8
	C305D	3.7	3.7	—	—	1.9 ± 0.8
High cresolase	C306D	2.2	—	—	55	0

TABLE V

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON 1.0 MG OF 3,4-DIMETHYL PHENOL

Reaction time, 250 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Type of enzyme	*Enzyme preparation No.*	*Cu in enzyme µg*	*Cu in effluent µg*	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
				catecholase activity	*cresolase activity*	
High catecholase	C312-2	4.5	4.8	—	—	15.4 ± 1.6
	C312-2	4.5	4.0	108	56	15.3 ± 2.1
	C313-1	3.6	3.8	91	76	15.2 ± 1.4
	C313-1	3.6	3.4	98	72	14.8 ± 1.4
High cresolase	C307D	3.7	3.3	51	80	4.5 ± 1.2
	C307D	3.7	3.7	—	—	5.5 ± 1.2

It will be noted that the amount of radiocopper incorporated into the enzyme was small, regardless of whether a high cresolase or a high catecholase preparation was used.

Exchange of enzyme copper during the oxidation of 3,4-dimethylphenol

In the presence of the large amount of enzyme used, the oxidation started immediately and the use of a primer was not necessary. The results, summarized in Table V, show that the high catecholase enzyme showed an approximately three times higher incorporation of radiocopper than the high cresolase enzyme.

Exchange of tyrosinase copper during the oxidation of D*-tyrosyl-*L*-alanine*

This dipeptide showed the same oxygen uptake as tyrosine (4 atoms/mole). Therefore, the oxidation probably followed a pathway similar to that proposed for tyrosine[8]. The results of a duplicate exchange experiment with this substrate are presented in Table VI.

TABLE VI

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING THE OXIDATION OF 2.0 MG OF L-TYROSYL-L-ALANINE

Reaction time, 100 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Enzyme preparation No (high catecholase)	*Cu in effluent µg*	*Cu in emzyme µg*	*% radioactivity incorporated into the enzyme*
C305D	3.7	—	32.6 ± 1.9
C305D	3.7	3.4	34.4 ± 2.0

DISCUSSION

The exchange experiments could not be carried out at the optimum substrate concentration for tyrosinase for the following reasons: (a) In order to have a measurable quantity of copper in the column effluents relatively large amounts of enzyme had to be employed; and (b) the amounts of substrates which could be used were limited by their low solubilities in water. Nevertheless, interesting speculations on the mode of action of tyrosinase can be based on the results obtained.

It should be remembered here that the data presented in Tables I–VI have been corrected by subtracting from the measured count the radioactivity incorporated into the resting enzyme (absence of substrate) under identical conditions[1]. As in the case of the resting enzyme[1], the copper exchange data obtained with catalytically functioning tyrosinase cannot be explained on the basis of enzyme inactivation. For example, the inactivation of the high catecholase enzyme C305D2 was in the same range whether 0.1 mg or 3.0 mg of 4-*tert.*-butylcatechol was oxidized (Table II) but the incorporation of radiocopper into the tyrosinase in these two cases was very different (0 % and 27.5 %, respectively).

The oxidation of 4-*tert.*-butylcatechol by a high catecholase enzyme resulted in a considerable incorporation of radiocopper into the protein when at least 1.0 mg of substrate was used. A parallel oxidation with a high cresolase preparation gave a much lower exchange (Table II). These findings are in line with the view that the monophenolase and *o*-dihydric phenolase activities of tyrosinase involve two different and distinct sites on the protein. The cresolase site cannot bring about the oxidation of a catechol (and the catecholase site cannot oxidize monohydric phenols). The high catecholase enzyme presumably had many more catecholase activity centers than the high cresolase preparation, even though the amount of enzyme copper involved was the same in both cases. The high catecholase enzyme, because it had more active catecholase sites, showed the highest extent of exchange with ^{64}Cu during the oxidation of 4-*tert.*-butylcatechol.

Regardless of the type of enzyme preparation involved, the oxidation of approx. 0.1 mg of 4-*tert.*-butylcatechol did not produce any measurable exchange of copper. In this case the ratio of substrate enzyme to molecules was approx. 300/1 while at the optimum substrate concentration this ratio is 1,000,000/1. Thus it may be that only a few of the catecholase activity centers participated in the oxidation of 0.1 mg of this substrate, and only a fraction of these actions may have led to the incorporation of radiocopper.

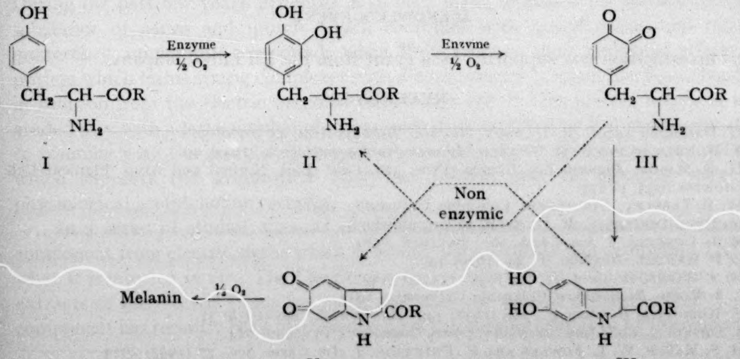
The experiments with 4,5-dimethylcatechol (Table III) supported the data obtained with 4-*tert.*-butylcatechol. For the same type of enzyme (high catecholase) and similar amounts of substrate (1.0 mg) the level of radiocopper incorporation into tyrosinase was comparable. This confirms the interpretation that the type of enzyme and total number of *o*-dihydric phenol molecules acted upon decide the magnitude of exchange.

During the tyrosinase catalyzed oxidation of 1.0 mg of 4-*tert.*-butylphenol (Table IV); little, if any, radioactive copper was incorporated into the enzyme, regardless of the type of tyrosinase used. In contrast, the oxidation of 1.0 mg of 3,4-dimethylphenol produced considerable copper exchange when a high catecholase preparation was used, and only small exchange when a high cresolase preparation was used (Table V). However, even in the case of the high catecholase enzyme catalyzed oxidation of 3,4-dimethylphenol, the copper exchange was only about one half of that observed when the same amount of 4,5-dimethylcatechol was the substrate. It seems attractive to ascribe the differences in copper exchange observed with the two monophenols as tyrosinase substrates to the fact that the 3,4-dimethylphenol is much more rapidly oxidized than 4-*tert.* butylphenol. Yet, a high cresolase preparation which oxidized 3,4-dimethyl phenol at least as fast, or faster, than a high catecholase enzyme, showed only a fraction of the radiocopper incorporation of the high catecholase enzyme. Thus, differences in rates of substrate oxidation do not appear to account for the exchange observations. Similar arguments seem to speak against the possibility that differences in the rates of oxidation of mono- and dihydric phenols account for the differences in copper exchange found.

It is known that the tyrosinase copper is necessary for cresolase activity. All experiments, so far, with the high cresolase enzyme resulted in a much lower exchange of copper than those involving high catecholase enzyme. These observations suggest that the copper atoms at the cresolase activity centers are non-exchangeable, *i.e.* only copper atoms at catecholase sites can be exchanged against ^{64}Cu. It also appears that catecholase activity centers are little or not at all involved in the oxidation of monohydric phenols or their oxidation product(s). At first, the relatively high exchange observed during the oxidation of 3,4-dimethylphenol by a high catecholase enzyme does not seem to fit the preceding suggestion. An attractive explanation for this "anomalous" case can be derived from a study by KENDAL[7]. He found that low concentrations of 3,4-dimethylphenol competitively inhibited the oxidation of 4,5-dimethylcatechol. Other similar substrate pairs (*e.g. p*-cresol and homocatechol) did not show this effect. KENDAL suggested that the "specific catecholase center" of the enzyme has a much greater affinity for 3,4-dimethyl phenol than for the other mono- or dihydric phenols. In our experiments, it appears possible that 3,4-dimethyl phenol formed many more complexes with catecholase activity centers when a high catecholase rather than high cresolase enzyme was used. If the exchange of copper is dependent on the formation of such complexes, our finding of higher exchange with high catecholase enzyme than with the high cresolase preparation when the dimethyl phenol was oxidized is understandable.

Anaerobic experiments might help to test the above suggestion. If complexing of the phenolic substrates with "catecholase sites" can indeed produce exchange, the presence of oxygen and the simultaneous oxidation of substrate may not be required.

The radiocopper incorporation into a high catecholase enzyme observed when it catalyzed the oxidation of the monohydric phenol, L-tyrosyl-L-alanine was of the same magnitude (Table VI) as that found with dihydric phenol as the substrate. This result disagrees with data obtained with 4-*tert.*-butyl phenol but can be explained by a consideration of the probable pathway of oxidation of the dipeptide. Presumably, L-tyrosyl-L-alanine (I; R = $NHCH(CH_3)COOH$) follows the oxidative reactions established for tyrosinase itself (I; R = OH) by RAPER[8]. As indicated in the reaction scheme below, an *o*-dihydric phenol (II) may form in this system by a non-enzymic reaction. Raper actually reacted synthetic dopaquinone (III; R = OH) with 5,6-dihydroindole-2-carboxylic acid (IV; R = OH) and found that dopa (II; R = OH) and hallachrome (V; R = OH) were formed. Thus it is quite possible that an *o*-dihydric phenol (II; R = $NHCH(CH_3)$–COOH) formed nonenzymically when L-tyrosyl-L-alanine was oxidized by tyrosinase. The considerable incorporation of ^{64}Cu into the enzyme during this reaction may be due to the oxidation of II (R = –NH–$CHCH_3$–COOH) at catecholase activity centers.



The results of the exchange experiments described above merit a reconsideration of the pathway of the tyrosinase catalyzed oxidation of monophenols. The current view that the oxidation of mono- and *o*-dihydric phenol involves two different sites on the enzyme, (with copper being essential to both activities) does not influence any evaluation as to whether a catechol is or is not the first product of the monophenol oxidation. Whether a catechol is the first intermediate of such oxidation at a monophenolase activity site and then migrates to a catecholase site for further oxidation to a quinone, or whether the production and oxidation of the catechol occurs at the same site, the oxidation of an equivalent amount of the corresponding catechol would be involved. Consequently, one would expect the same degree of exchange with ^{64}Cu during the enzymic oxidation of a monophenol and the corresponding *o*-dihydric phenol. In general, however, the experimental data indicated that considerable copper exchange occurred during the tyrosinase catalyzed oxidation of an *o*-dihydric phenol and little exchange took place during the oxidation of monohydric phenols. Therefore, the possibility is suggested that the oxidation of monohydric phenols by tyrosinase may not involve the corresponding *o*-dihydric phenols *per se* as an intermediate. Rather the monophenol might be directly converted to the corresponding *o*-benzoquinone.

KUBOWITZ[10] established that the copper bound to tyrosinase is reduced from the cupric to the cuprous state during the oxidation of *o*-dihydric phenols. Several authors[11–13] have suggested that the monophenolase activity of tyrosinase involves cuprous copper. These hypotheses were based on the observed elimination of the induction period apparent in monophenol oxidation by small amounts of reductants such as catechol and ascorbic acid. MASON[13] believes that a hydroxylation of monophenol to *o*-dihydric phenol occurs and produces an oxidation of cuprous to cupric enzyme copper. Again, the exchange experiments described here generally showed considerable radioactive copper incorporation into tyrosinase during the oxidation of *o*-dihydric phenols and minor copper exchange during the oxidation of monophenols. These findings are difficult to reconcile with MASON's hypothesis unless cupric ions cannot reach the enzyme activity sites. A configuration accessible to oxygen molecules and substrate but not to cupric ions would be required.

ACKNOWLEDGEMENT

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THE INTERACTION OF QUATERNARY AMMONIUM COMPOUNDS WITH HYALURONIC ACID

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SUMMARY

The interaction of *d*-tubocurarine (curare) and stilbamidine with hyaluronic acid has been investigated at pH 7.5, ionic strength 0.1. Curare fails to bind to the polysaccharide while strong binding of stilbamidine is observed. This observation, in conjunction with others, support the view that this acidic polysaccharide is not essential for the pharmacological effects of curare on nerve impulse conduction. Considerable binding of curare and other quaternary ammonium compounds is observed in distilled water and is evidently due to the Donnan effect.

Lack of binding of curare to hyaluronic acid may be due to the fact that unlike stilbamidine the inter-quaternary distance is not complementary to the inter-anionic distance of the polysaccharide. Since stilbamidine and curare have been considered to have the same interquaternary distance—14 Å—the present findings raise the question whether current views on the configuration of the curare molecule may have to be revised.

INTRODUCTION

During the past few years, attempts have been made to isolate the specific receptor substance of nerve and muscle which combines with acetylcholine and related quaternary ammonium compounds when they produce their biological effects. A protein which forms strong complexes with *d*-tubocurarine (curare) has been obtained in solution from the electric organ of the electric eel[1-3]. This protein interacts in a graded way with a large number of compounds demonstrated by Nachmansohn *et al.* to combine with the acetylcholine system[4]. Considerable evidence has accumulated which supports the assumption[3] that this protein may be identical with the physiological acetylcholine receptor.

In a series of studies, Chagas *et al.* have reported the isolation of a different component from electric tissue which combines with a curare-like compound TRIEG when it is injected *in vivo*. The TRIEG remains associated with the component when extracts of the electric tissue are made and dialyzed against distilled water[5-8]. The component has recently been identified as a polysaccharide similar to hyaluronic acid[8].

Abbreviations: TRIEG, triethiodide of gallamine; PAM, pyridine-2-aldoxime methiodide.

In view of the highly anionic character of this macromolecule, it appeared interesting to examine the nature of its binding properties more closely under conditions other than distilled water. Accordingly, the interaction of curare and a number of other quaternary compounds with hyaluronic acid has been investigated in salt solution at neutral pH. Using equilibrium dialysis, it was found that hyaluronic acid fails to interact with curare at pH 7.5, ionic strength 0.1. On the other hand, considerable binding of curare and other quaternary compounds is observed in water; this may be attributed primarily to the Donnan effect.

These studies, in conjunction with others previously reported[9, 10] have led to interesting problems about the configuration of the curare molecule. Some speculations along these lines will be presented in this paper.

MATERIALS AND METHODS

Hyaluronic acid, obtained from the Mann Research Laboratories, was about 50 % pure on the basis of the Dische carbazole reaction. *d*-Tubocurarine, acetylcholine and benzoylcholine were obtained from the K & K Chemical Company. Stilbamidine was a product of Merck Chemical Co. PAM, hexamethylene bis-PAM and decamethylene bis-PAM were synthesized by Dr. SARA GINSBURG of this laboratory.

The concentration of acetylcholine was determined by HESTRIN's method[11], that of the other compounds spectrophotometrically. Some of their u.v. characteristics have been presented elsewhere[3, 10].

Equilibrium dialysis experiments were carried out as previously described[2, 3, 10]. It was necessary to use tris buffer since stilbamadine is quite insoluble in phosphate.

Dialysis experiments in distilled water were carried out in a manner similar to that described by CHAGAS *et al.* The hyaluronic acid was dialyzed first against dilute NaCl then against distilled water. The polysaccharide was mixed with the various quaternary compounds at concentrations about 10 times greater than required to neutralize all the negative charges in the polysaccharide. The mixtures were dialyzed against several changes of distilled water until the dialyzates were essentially free of u.v. absorbing material. The concentration of quaternary compound and hyaluronic acid within the bags was then determined.

RESULTS AND DISCUSSION

The binding of curare and stilbamidine to hyaluronic acid at pH 7.5, ionic strength 0.1 is shown in Fig. 1. It is apparent that under these conditions curare failed to interact with the polysaccharide. Strong binding of stilbamidine was observed.

The fact that hyaluronic acid does not combine with curare in dilute salt solution at pH 7.5 makes it likely that this macromolecule does not play an important role as a physiological receptor for curare and related compounds in the sense defined above. The behavior of this polysaccharide may be contrasted with that exhibited by the receptor protein isolated from the electric organ which combines very strongly with curare under these conditions[1–3]. The present result clearly shows that binding of curare to the receptor protein is indeed unique and not merely the result of non-specific coulombic interaction. Nevertheless, the complete absence of binding of a diquaternary compound like curare to the highly anionic polysaccharide was somewhat unexpected and may have considerable bearing on the configuration of the curare molecule.

The strong binding of stilbamidine is in contrast to that of curare (Fig. 1). Moreover, stilbamidine quantitatively precipitates hyaluronic acid[12] with an equivalence ratio of one stilbamidine for two negative charges. It has been shown[10, 12] that this kind of interaction of a rigid diquaternary compound such as stilbamidine may be used as the basis for measuring the inter-anionic distance of the repeating unit of a macromolecule, *e.g.*, chondroitin sulfate. Thus it would appear that the inter-anionic distance in hyaluronic acid is fairly close to 14 Å, the inter-quaternary distance in stilbamidine. The distance between positive charges in curare has also been considered to be about 14 Å. However, the fact that curare fails to interact with hyaluronic acid as well as the marked differences in the combination of curare and stilbamidine with a variety of other macromolecules[12] lead to the speculation that the inter-quaternary distance in curare may be significantly less than 14 Å. Such a conclusion is particularly interesting in view of the report by LOEWE AND HARVEY[13] that a model of curare may be made in which the cationic groups are separated by only 6.5 Å. If this premise is correct, the lack of binding of curare to hyaluronic acid would be explained by the fact that the anionic groups are at a distance which is far too great to accommodate the curare molecule; conversely, this finding supports the notion that the inter-quaternary distance may be considerable less than 14 Å. For more definitive evidence, it is clear that other types of experiments, such as X-ray crystallography of the curare molecule are required.

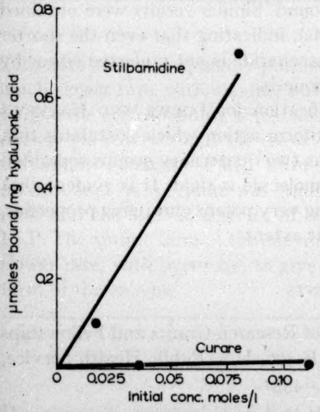

Fig. 1. Comparison of binding of stilbamidine and curare to hyaluronic acid. pH 7.5, $\mu = 0.1$ (Tris), 0°.

TABLE I

RETENTION OF VARIOUS QUATERNARY AMMONIUM COMPOUNDS BY HYALURONIC ACID IN DISTILLED WATER

Compound	*moles "bound" per mole hexuronic acid*
Curare	0.50, 0.43
Stilbamidine	0.31, 0.23, 0.35
Acetylcholine	0.37
Benzoylcholine	0.50
Hexamethylene bis-PAM	0.43
Decamethylene bis-PAM	0.43
PAM	0.36

It is apparent that in distilled water the interaction of curare and other quaternary compounds observed (Table I) is non-specific, *i.e.*, no distinction in binding is made between a variety of cationic substances. Certainly the degree of binding in no way paralleled the affinity of these compounds for the physiological receptor as determined by experiments on the intact cell[14, 15]. Retention of these compounds most likely results from the Donnan membrane effect rather than from true complex formation. However, it is to be noted that in no instance was the amount of quaternary compound retained sufficient to completely neutralize all the charges on the hyaluronic acid. This is partly explained by the fact that the final pH inside the dialysis bags was fairly low and thus some of the carboxyl groups were in the unionized state. Even if this were the case, the interesting point is that the amount of di-quaternary and mono-quaternary retained was almost the same. This shows that in distilled water, where repulsion of similar charges of a flexible molecule should be at a maximum, the fully extended form did not seem to predominate. Thus in decamethylene bis-PAM the maximum distance between cationic groups is approx. 14 Å; if the fully extended configuration occurred to a significant extent the compound could combine simultaneously with two negative charges in hyaluronic acid as is the case with stilbamidine. It would be expected that the number of moles of this compound bound would be significantly less than the mono-quaternary, PAM; this was not found. Similar results were obtained with chondroitin sulfate (unpublished experiments), indicating that even the shorter distance separating negative groups of this polysaccharide is not preferred either by the hexa- or decamethylene bis-quaternary compounds.

These results appear to provide some justification for LOEWE AND HARVEY's objections[13] to the equidistance concept of curariform action which postulates that strong curare action results when a compound has two quaternary groups separated by 14 Å. This situation pertains only when the molecule is rigid. It is evident that decamethylene bis-PAM, a flexible molecule having very potent curarizing properties, fails to attain the 14-Å distance to any significant extent.

ACKNOWLEDGEMENTS

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ON THE MECHANISM OF ACTION OF GLUTAMIC-ASPARTIC TRANSAMINASE: INTERMEDIATE STEPS IN THE REACTION*

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SUMMARY

Substrate amounts of highly purified GAT react with glutamate, aspartate and, at a much slower rate, with alanine, but not with glycine, arginine, serine and isoleucine, to give the corresponding ketoacids. The absorption spectrum of the enzyme changes during the reaction and the coenzyme passes from the aldehydic (enzyme–PLP) to the aminic (enzymic–PMP) form. Enzyme-PMP was isolated from the reaction mixture and its spectrum was studied at different pH values. The coenzyme/protein ratio and the specific activity of enzyme–PMP were the same as those of enzyme–PLP. The aminic form of the enzyme reacts with ketoglutarate, oxalacetate and, at a slower rate, with pyruvate, to give the corresponding amino acids and the aldehydic form of the enzyme.

INTRODUCTION

It is generally assumed (1–4) that the mechanism of enzymic transamination may be represented by the following equations:

$$\text{Enzyme–PLP} + \text{amino acid I} \leftrightharpoons \text{enzyme–PMP} + \text{keto acid I} \qquad (1)$$

$$\text{Enzyme–PMP} + \text{keto acid II} \leftrightharpoons \text{enzyme–PLP} + \text{amino acid II} \qquad (2)$$

This hypothesis was first substantiated by SNELL *et al.*[4] in their extensive work on model reactions and it has been recently confirmed by studies on purified enzymes. MEISTER *et al.*[5] showed that both PLP and PMP can activate the GAT system. However, most of the attempts to demonstrate the conversion of enzyme–PLP to enzyme–PMP were not successful[3] until JENKINS *et al.*[6,7] not only confirmed the presence of PLP in GAT from pig heart, but, in a preliminary note[8], reported also that, after the addition of glutamate to the enzyme, the prosthetic group was transformed into PMP, and that the reaction was reversed by an excess of α-ketoglutarate.

Abbreviations: GAT, glutamic–aspartic transaminase; MDH, malic dehydrogenase; LDH, lactic dehydrogenase; enzyme–PLP, aldehydic form of transaminase; enzyme–PMP, aminic form of transaminase; PLP, pyridoxal-5-phosphate; PMP, pyridoxamine-5-phosphate; DPNH, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide.

[*] A preliminary report of the present work was read on May 23rd 1959 at the V Jornadas Bioquimicas Latinas (Barcelona).

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In the work here described the reaction of highly purified GAT with several amino acids was investigated. Moreover, enzyme–PMP was isolated, so that it has been possible to study also the reaction of enzyme–PMP with oxalacetate, ketoglutarate and other keto acids.

EXPERIMENTAL

Materials

γ-amino acids were from Merck and Co., Darmstadt (Germany); oxalacetic acid from California Foundation for Biochemical Research, Los Angeles (U.S.A.); α-ketoglutaric acid from Sigma Chemical Co., St. Louis (U.S.A.); PLP from Hoffmann-La Roche, Basel (Switzerland); PMP was prepared according to PETERSON AND SOBER[9].

GAT was prepared from pig heart according to LIS[10] with minor modifications[11]. MDH was prepared together with GAT[11]. LDH and DPNH were from Böhringer and Sohn (Germany).

Methods

Activity measurements: GAT activity was determined as previously described[10] with the minor modification that the 10-min incubation of the aminoacid and enzyme before the addition of the keto acid was omitted.

Proteins were determined by the biuret method[12] and by their absorbancy at 280 mμ in 0.1 M phosphate buffer (pH 7), after having determined the specific extinction coefficient at that wavelength by reference to the dry weight of the protein, after heating at 110° in the air until constant weight is reached.

Determination of coenzymes: Coenzymes were removed from the protein by solving the enzyme in 0.1 N NaOH or KOH. For the qualitative identification of both forms of the coenzyme, the KOH solution of the enzyme was neutralized with $HClO_4$; the precipitated $KClO_4$ and denatured protein were removed by centrifugation and the supernatant was lyophilized, dissolved in a minimum of water and analysed by paper chromatography[13] and electrophoresis[14].

PLP was quantitatively determined on the basis of its absorption at 388 mμ in the 0.1 N NaOH solution[6,15]. For the quantitative determination of PMP the 0.1 N NaOH solution of the enzyme was brought to pH 4.6 with acetic acid. This caused the precipitation of denatured protein, which was removed by centrifugation; the clear supernatant fluid was brought to pH 3.2 with 1 M citric acid and placed on the top of a Dowex 50 column, 200–300 mesh, (4 × 0.7 cm) in the Na^+-form, equilibrated with citrate buffer (pH 3.2) 0.1 M respecting Na^+. The column was washed with 15 ml of the same citrate buffer. PMP was eluted from the column in 0.1 M sodium acetate. PMP was usually completely recovered in the first 5 ml of the elution fluid; this was successively brought to pH 7 and used for the fluorometric determination of PMP in a Farrand spectrofluorometer, using an exciting light at 330 mμ and analysing at 390 mμ as previously described[16]. Controls of the chromatographic behaviour and recovery were made with pure PMP, both alone and added to the enzyme extract.

Preparation of enzyme–PLP: A solution of the enzyme obtained according to LIS[10,11] was added, with ketoglutarate, to a final concentration of 0.01 M, brought to pH 5.4 with 1 N acetic acid and precipitated in the cold with 1 volume of acetone. After centrifugation the supernatant was discarded and the enzyme was dissolved in 0.01 *M* acetate buffer (pH 5.4) in a volume corresponding to one half of the original solution; the enzyme was then again precipitated with acetone. This treatment was repeated twice in order to remove the remaining traces of the keto acid. Acetone was eliminated from the final precipitate by gentle aeration in the cold. The protein was then dissolved in the desired buffer at the desired concentration and the solution obtained was centrifuged again in order to remove traces of insoluble material.

Reaction between enzyme–PLP and amino acids

Reaction mixture: To a solution of enzyme–PLP in 0.08 *M* pyrophosphate buffer (pH 8.4), a 1 *M* amino acid solution (natrium salt) in the same buffer was added to a final concentration of 0.3 *M*. The final concentration of the enzyme was 0.5%. Sparingly soluble amino acids (*i.e.* serine, isoleucine, arginine) were added as saturated solutions to a final concentration of 0.02 *M*. The absorption spectra of the solution were determined before and after the addition of the amino acid.

In experiments for the quantitative determination of oxalacetate and pyruvate, the reaction was carried out in 0.02 *M* phosphate buffer (pH 7.4).

Determination of oxalacetate: For the determination of oxalacetate, the MDH system proposed by KARMEN[15] was used. To the enzyme–PLP–aspartate reaction mixture was added 1/60 volume of the MDH solution (0.1 mg protein/ml) and 1/60 volume of the DPNH solution (5 µmoles/ml). A blank was carried out containing all the components excepting GAT. The increase of the absorbancy at 340 mµ upon addition of DPNH was measured in the test and blank solutions by means of a Beckman D.U. Spectrophotometer. The amount of oxalacetate was calculated from the difference in the increase of absorbancy in the test and blank. A preliminary control had shown that no oxalacetate was formed when aspartate was omitted from the reaction mixture.

Determination of pyruvate: Pyruvate was determined in the GAT–alanine reaction mixture by the method described by BAUER[18]. The experimental procedure was the same as the one described above for oxalacetate, excepting that MDH was replaced by LDH.

Determination of other ketoacids: The enzyme was precipitated with acetone from the enzyme–PLP–glutamate (or other amino acid) reaction mixture and the keto acid was determined in the supernatant by the method of CAVALLINI AND MONDOVI[19] after removal of acetone by distillation under vacuum.

Preparation of enzyme–PMP: The enzyme was recovered from the GAT–glutamate (or aspartate) reaction mixture by precipitation with acetone at pH 5.4. The precipitate was dissolved in a volume of 0.01 *M* acetate buffer (pH 5.4) equal to that of the original solution and precipitated with 1 volume of acetone. The treatment was repeated twice in order to remove the remaining traces of the amino acid. The final precipitate was gently aerated in the cold so as to remove acetone, and then solved in the desired buffer.

Reaction between enzyme–PMP and ketoacids

0.05 *M* ketoacid (sodium salt, in 0.08 *M* pyrophosphate buffer (pH 8.4) was added to the enzyme–PMP solution in a final concentration of 0.01 *M* ketoacid and 0.5 % protein.

The absorption spectrum was recorded before and after the addition. After 10 min at room temperature, the enzyme was precipitated with acetone; the supernatant solution was concentrated under vacuum and the amino acids present in it were determined according to ASPEN *et al.*[20].

RESULTS AND DISCUSSION

Coenzyme content, specific activity and absorption spectra

The coenzyme content and specific activity of the GAT preparation, obtained by the method described by LIS[10,11], of enzyme–PLP and of enzyme–PMP are reported in Table I. As this table shows, some PMP (about 8 % of the total coenzyme) is present in the GAT preparation obtained from the electrophoresis column[10,11]. Because no ketoacid or aminoacid was added during the preparation and, because, under the experimental conditions followed, the two forms are about equally stable, it can be assumed that this is the ratio in which the two forms of the enzyme occur in pig heart.

Only a little PMP remains in the enzyme preparation after treatment with one of its keto acid substrates, while no PLP can be detected in enzyme–PMP. When, however, these data are considered, it should be borne in mind that the fluorometric method employed for the determination of PMP is far more sensitive than the spectrophotometric method employed for PLP, so that, if enzyme–PMP contained traces of PLP, they would escape detection. The coenzyme/protein ratio found for our preparation (17.4 μmole/g protein) is in good agreement with data calculated by JENKINS *et al.*[6] (13.4 to 14.7 μmole/g protein), on the assumption, made by those authors, that their preparation is 81 % pure.

The specific activity of the three enzyme preparations is essentially the same in both directions.

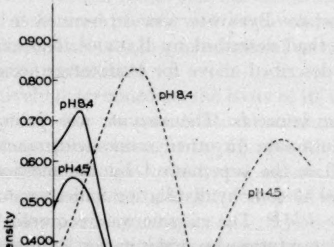

Fig. 1. Absorption spectra of enzyme–PLP (-----) and of enzyme–PMP (——) in 0.1 M pyrophosphate buffer (pH 8.4) and in 0.1 M acetate buffer (pH 4.5). All solutions contained 6 mg of protein /ml.

The absorption spectra of enzyme–PMP and of enzyme–PLP at different pH values are shown in Fig. 1. The pH effect on the spectrum, which is marked on enzyme–PLP[7], is, on enzyme–PMP, very modest.

Reaction between enzyme–PLP and aminoacids

When glutamate or aspartate is added (to a final concentration of 0.3 *M*) the absorption peak of enzyme–PLP at pH 8.4 in the upper u.v. region shifts from 360 to 335 mμ. A similar shift was observed by JENKINS AND SIZER[8] upon addition of glutamate (to a final concentration of 0.5 *M*) to their GAT preparation. Such changes do not occur when glutamate or aspartate are replaced by amino acids on which the enzyme is not active, *i.e.* glycine, isoleucine, serine and arginine. Table II records some quantitative aspects of the reaction between enzyme–PLP and several amino acids.

It is evident from Table II that, when glutamate or aspartate is added to enzyme–PLP to a final concentration of 0.3 *M*, ketoglutarate or, alternatively, oxalacetate appears in the reaction mixture in amounts approximately equivalent to the number of coenzyme molecules bound to the enzyme. The determination of the coenzyme after the addition of either of these amino acids showed that PLP had completely disappeared and that an equivalent amount of PMP had been produced.

TABLE I

SPECIFIC ACTIVITY AND COENZYME CONTENT OF A TYPICAL PREPARATION OF GAT PREPARED ACCORDING TO LIS[10,11], OF ENZYME–PLP AND OF ENZYME–PMP

	PLP (μmoles/g protein)	*PMP (μmoles/g protein)*	*g protein/mole total coenzyme (PLP + PMP)*	*Specific activity (units/mg protein)*	
				Aspartate + ketoglutarate	*Glutamate + oxalacetate*
Enzyme preparation according to LIS[10,11]	15.6	1.4	58,800	5,600	12,000
enzyme–PLP	17.2	0.28	57,200	5,600	11,900
enzyme–PMP	none	17.4	57,400	5,700	12,000

TABLE II

TRANSAMINATION BETWEEN ENZYME–PLP AND AMINO ACIDS

All data are referred to 1 ml of reaction mixture containing 5 mg of enzyme–protein.

μmoles enzyme bound PLP before addition of amino acid	*μmoles amino acid added*	*after addition of the amino acid*		
		μmoles keto acid formed	*μmoles enzyme–PLP*	*μmoles enzyme–PMP*
0.085	300 (glutamate)	0.082 (ketoglutarate)	0.000	0.087
0.087	300 (aspartate)	0.084 (oxalacetate)	0.000	0.084
0.084	20 (aspartate)	not determined	0.046	0.043
0.086	20 (aspartate)*	0.089	0.000	0.088
0.087	300 (alanine)	0.083 (pyruvate)	0.000	0.081
0.086	300 (serine)	none	0.084	
0.086	20 (isoleucine)	none	0.081	
0.085	20 (arginine)	none	0.085	

* in the presence of MDH and DPNH at pH 7.4.

With lower concentrations of the amino acid (*e.g.* 0.02 *M*) both PLP and PMP were found in the alkaline extract of the enzyme. It is of interest to observe that, even with this concentration of glutamate or aspartate, the change of the absorption spectrum at pH 8.4 was complete (*i.e.* identical with the one obtained with 0.3 *M* amino acid). These findings might be interpreted by assuming that the equilibrium of the reaction is pH-dependent: when, that is to say, the pH of the reaction mixture is adjusted to 5.4 in order to precipitate the enzyme with acetone, a part of the keto acid formed at pH 8.4 might react with enzyme–PMP to reconstitute enzyme–PLP and the amino acid.

This hypothesis is in agreement with the observation made by JENKINS[7] that, when the pH is lowered, enzyme–PLP is transformed into the non-active form, which would be subtracted from the equilibrium. Further evidence in support of this interpretation is provided by our finding that, when the reaction of enzyme–PLP with aspartate is carried out at pH 7.4 in the presence of the MDH system (which converts oxalacetate into malate as soon as it is formed and thus subtracts it from the equilibrium) no PLP is found, even when low concentrations of aspartate (0.02 *M*) are used, in the alkaline extract of the enzyme recovered from the reaction mixture by precipitation with acetone at pH 5.4.

It has been mentioned above that some amino acids, on which GAT is not active (*i.e.* serine, glycine, isoleucine and arginine), were unable to modify the spectrum of enzyme–PLP. Their incapacity to react with GAT was confirmed by the finding that, when they were added to the enzyme solution, enzyme-bound PLP was not transformed into PMP and that no keto acid appeared in the reaction mixture (see Table II).

In this connection we have thought it interesting to investigate the behaviour of alanine, which, according to CAMMARATA AND COHEN[21], undergoes transamination with ketoglutarate in the presence of a GAT preparation from pig heart; alanine, however, transaminates at a much lower rate than aspartate. The attribution of this effect to the presence of contaminating traces of glutamic–alanine transaminase could not be excluded. In the present research it was found that, when alanine was added to enzyme–PLP, spectral changes occurred, which were identical with those observed with glutamate and aspartate. Pyruvate, moreover, appeared in the reaction mixture and the enzyme-bound vitamin B_6-derivative turned from PLP into PMP (see Table II).

The reaction of enzyme–PLP with alanine apparently takes place more slowly than the one with aspartate or glutamate, because, when low concentrations of the amino acid (0.02 *M*) are used, the changes in the absorption spectrum, which are instantaneous with glutamate or aspartate, take place so slowly that they can be followed in the spectrophotometer (Table IV).

Reaction between enzyme–PMP and keto acids

When ketoglutarate or oxalacetate was added, the absorption spectrum of enzyme–PMP at pH 8.4 shifted back from a spectrum with a peak at 335 mμ to one with a peak at 360 mμ.

Some quantitative aspects of this reaction are reported in Table III. As this table shows, it was possible to recover glutamate or, alternatively, aspartate from the reaction mixture in amounts equivalent, within the limits of experimental error,

TABLE III

TRANSAMINATION BETWEEN ENZYME–PMP AND KETO ACIDS

All data are referred to 1 ml of the final reaction mixture containing 5 mg enzyme protein.

µmoles enzyme bound PMP before the addition of keto acid	µmoles keto acid added	after addition of the keto acid		
		µmoles amino acid formed*	µmoles enzyme-PMP	µmoles enzyme-PLP
0.088	10 (ketoglutarate)	0.091 (glutamate)	0.012	0.081
0.083	10 (ketoglutarate)	0.079 (glutamate)		0.087
0.084	10 (oxalacetate)	0.095 (aspartate)	0.011	0.085
0.082	10 (oxalacetate)	0.073 (aspartate)		0.088
0.087	10 (pyruvate)	0.072 (alanine)		0.083

* The method for the determination of amino acids gave a standard deviation of ± 9%.

TABLE IV

REACTION BETWEEN ENZYME–PLP (0.38%) AND 0.02 *M* L-ALANINE IN 0.09 *M* PYROPHOSPHATE BUFFER (pH 8.4)

Absorbances of the reaction mixture	Time after addition of alanine						
	0 sec	120 sec	240 sec	360 sec	480 sec	600 sec	5,000 sec
360 mµ	0.530	0.508	0.466	0.406	0.356	0.328	0.260
335 mµ	0.325	0.338	0.360	0.389	0.415	0.430	0.460

to the number of coenzyme molecules bound to the enzyme. The determination of vitamin B_6-derivatives extracted from the enzyme revealed the presence of PLP and of traces only of PMP. The coenzyme/protein and activity/protein ratios of the enzyme isolated from the reaction mixture correspond to those found for enzyme–PLP.

As Table III shows, enzyme–PMP can react with pyruvate to give enzyme–PLP and alanine. This reaction is not immediate; it requires several minutes to reach completion. These latter results, together with those previously reported for the reaction of enzyme–PLP with alanine, suggest that GAT from pig heart is itself active also on alanine and pyruvate; the reaction of the enzyme with these substrates is much slower than the one with glutamate, aspartate, ketoglutarate or oxalacetate.

Taken as a whole, the results here described provide further evidence in support of the two steps by which enzymic transamination is postulated to occur.

ADDENDUM

When this manuscript was ready for publication, a paper on the same subject by T. W. JENKINS *et al.*[22] appeared. Our investigation of the reaction of GAT with each of its substrates gave results that are in substantial agreement with those obtained by these American authors on a different enzyme preparation and with different methods.

The results here described, moreover, provide original information about the reaction of the enzyme with non-substrate amino acids and with pyruvic acid; and these contribute to our knowledge of the specificity of the active site of the enzyme.

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THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

V. CHROMATOGRAPHIC ANALYSIS OF THETIN–HOMOCYSTEINE METHYLPHERASE ON MODIFIED CELLULOSE COLUMNS

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SUMMARY

The chromatographic behavior of the enzyme thetin–homocysteine methylpherase has been examined on both DEAE-cellulose and CM-cellulose columns. The enzyme is not held on DEAE-cellulose, but this adsorbent has been found suitable for a final purification step. After DEAE-cellulose treatment, thetin–homocysteine methylpherase behaves as a single component during chromatography on CM-cellulose columns. It has been shown that there is no chromatographic difference between the monomer and the polymeric form of the enzyme. On the other hand, chromatographic evidence has been presented for the formation of a number of mixed disulfides of thetin–homocysteine methylpherase and various low molecular weight thiols. Such mixed disulfides often exhibit markedly altered chromatographic behavior and may be of some general significance.

INTRODUCTION

The enzyme THMenz has been purified from horse liver to the point where conventional protein fractionation techniques are not capable of effecting further changes in the composition of the preparation[1]. In view of the fact that THMenz accounts for 1 % of the protein of horse liver, it was considered of interest to study the chromatographic behavior of the preparation in order to obtain further information on the state of purity of the enzyme. It had been found that THMenz can, under very mild conditions, undergo a reversible polymerization reaction. The aggregates formed are unusual in that they are linked by disulfide bonds and not simply by secondary valence forces[1,2]. It was therefore also of interest to study the chromatographic behavior of the polymeric** form of the protein in comparison with that of the monomer.

Abbreviations: THMenz, thetin–homocysteine methylpherase; DEAE, diethylaminoethyl; CM, carboxymethyl.

* Twenty-first paper in a series on enzymic mechanisms in transmethylation.

** The polymeric form of THMenz invariably consists of a mixture of molecular species ranging from monomer to aggregates containing 50 or more monomer units. The composition of the mixture with respect to any one component is defined by the degree of polymerization in the manner predicted for a trifunctional monomer by the statistical theory of Stockmayer[4].

Protein chromatography on the modified cellulose columns of PETERSON AND SOBER[3] has found wide application in recent years. In the present study, both DEAE-cellulose and CM-cellulose columns were employed. Conditions suitable for the chromatographic analysis of THMenz were found with CM-cellulose as the absorbent, and no evidence of chromatographic heterogeneity of the unmodified enzyme was found. Furthermore, it has been found that monomer and polymer preparations of the enzyme behave identically during chromatographic analysis. During the course of these studies, a series of observations were made of the chromatographic behavior of mixed disulfides of THMenz with low molecular weight thiols. In some cases, such mixed disulfides were found to have markedly altered chromatographic behavior. It is believed that such results may be of some general significance.

MATERIALS AND METHODS

THMenz was prepared as previously described[1], with the modifications introduced in a more recent publication from this laboratory[2]. Electrophoresis had been previously used after the ammonium sulfate step to remove a small amount of contaminating, non-protein material. It has now been found to be more convenient to remove this substance by means of treatment with DEAE cellulose as described in the present communication. Enzyme activity was measured as previously described[1], with dimethylacetothetin serving as the methyl donor. Protein concentrations were determined by measuring the absorbance at 280 mμ in a Beckman model DU spectrophotometer using 13.0 as the extinction coefficient for a 1 % solution[4].

DEAE cellulose was obtained from Eastman Organic Chemicals and washed according to the directions of PETERSON AND SOBER[3]. CM cellulose was prepared from Solka-Floc according to PETERSON AND SOBER, except that the cellulose powder was not sieved before use. All other reagents were commercial products of the highest available purity.

Prior to chromatographic analysis, the enzyme preparations were dialyzed *versus* 1000 volumes of a buffer solution of the same composition as the starting buffer to be employed in the subsequent chromatography. In general, dialysis for a period of 4 h at 2° was found to be satisfactory, although a number of experiments were performed which involved longer dialysis. The enzyme preparations used were either polymer preparations, or monomer prepared as follows: the protein was first depolymerized by treating a 1-% solution at pH 7.4 with 2 mg/ml of homocysteine for 15 min at 37° and then dialyzed for 4 h in the buffer to be used in the subsequent chromatography, which also included 0.005 *M* thioglycollate. Under these conditions, no appreciable amounts of mixed disulfides were found on chromatography. In experiments designed to demonstrate the occurrence of mixed disulfides of the protein with the thiol of the buffer, dialysis was carried out for periods of at least 12 h.

The columns were packed with the adsorbent after titration to the proper pH and equilibration with the starting buffer. Packing was accomplished by gravity followed by the application of a pressure of seven pounds/in². All experiments were performed at 2°, and constant volume fractions were collected with the aid of a GME fraction collector.

RESULTS

Many proteins with isoelectric points in the neutral or acidic pH range have been successfully studied by chromatography on DEAE cellulose[5,6]. Since it has been shown that THMenz exhibits an isoelectric point of 6.4 in 0.05 *M* phosphate buffer[1], it was felt that this protein too should be amenable to chromatographic analysis using DEAE cellulose as the adsorbent. It was found, however, that THMenz is not adsorbed on DEAE cellulose under any conditions and that the activity may be quantitatively recovered as a sharp band emerging at the column front (Fig. 1). The chromatogram illustrated in Fig. 1 also shows that a second peak of u.v. absorbing material is eluted at a higher ionic strength. This material corresponds to the relatively acidic electrophoretic impurity observed by DURELL *et al.*[1] to be present in the purified enzyme preparation and is non-protein in nature. In the light of this observation THMenz preparations were routinely purified by passage through a DEAE column before further studies were carried out. The experiments reported in this paper were performed with such material unless otherwise stated. The chromatographic behavior of the enzyme was found to be unchanged after passage through DEAE. It will be seen below, however, that the chromatographic behavior of the protein can be greatly altered by chemical modification with certain thiols, and that some mixed disulfides of THMenz may be readily chromatographed on DEAE cellulose.

In view of the fact that the unmodified protein is not held on DEAE cellulose columns, the behavior of the enzyme was studied on columns of CM cellulose. It was found that the enzyme was readily adsorbed onto such columns and quanti-

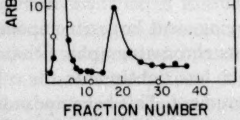

Fig. 1. The elution pattern of THMenz on DEAE cellulose. The starting solvent was 0.005 *M* in both phosphate and thioglycollate, and 0.001 *M* in Versene at pH 6.8. At Fraction 11, the buffer was changed to 0.4 *M* phosphate, 0.4 *M* sodium chloride and 0.001 *M* Versene. Activity and protein concentrations are expressed in arbitrary units. The dimensions of the column were 1 × 15 cm and the fraction size 2 ml.

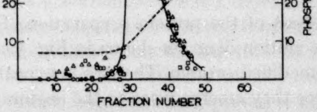

Fig. 2. Elution patterns of monomer and polymer on CM cellulose columns. The dimensions of the column were 1 × 15 cm, 10-mg samples were applied, and 2-ml fractions were collected. This figure is the superposition of two separate experiments performed under identical conditions. The activity and protein concentration figures are expressed in arbitrary units, normalized at the peak maximum. The initial buffer was 0.005 *M* in phosphate and 0.001 *M* in Versene at pH 6.1. In the case of the monomer, the buffer also included 0.005 *M* thioglycollate. The gradient was obtained by the addition of a buffer at pH 7.6 which is 0.1 *M* in phosphate to the starting buffer in a mixing chamber.

tatively eluted as a single peak by means of either sodium chloride or potassium phosphate gradients. The normalized results of two separate experiments with identical CM cellulose columns are shown in Fig. 2. In these experiments, the samples differed in that one was highly polymerized while the other was a monomer preparation. In the case of the monomer, the buffer also contained 0.005 *M* thioglycollate. It is readily seen that the two preparations exhibit identical chromatographic behavior, and that there is no evidence for the presence of appreciable amounts of extraneous material in the enzyme preparations. A number of similar experiments, under varying conditions, were performed in an unsuccessful attempt to resolve chromatographically the components of the polymeric mixture.

Mixed disulfides

Although THMenz normally behaves as a single substance on CM cellulose chromatography, it is possible to induce chromatographic heterogeneity by means of small changes in experimental procedure which result in readily reversible chemical modifications of the enzyme. THMenz polymer preparations exhibit only a low solubility in the dilute buffers employed in CM cellulose chromatography, but the solubility of the enzyme is appreciably enhanced by depolymerization of the protein. Accordingly, THMenz preparations are routinely depolymerized before preparative scale chromatography on CM-cellulose columns. When dialysis of the monomer against the column buffer containing 0.005 *M* thioglycollate is allowed to proceed overnight rather than the usual 4 h, chromatography on CM cellulose shows the presence of a number of components. This situation is illustrated in Fig. 3, in which three enzymically active components are seen to be present. In this chromatogram, the first peak also contains some inactive material, since this protein preparation had not previously been purified either electrophoretically or by DEAE chromatography. Each of the three components seen in the elution diagram was collected as an ammonium sulfate precipitate and depolymerized and dialyzed for the usual 4 h. On rechromatography, each of the three components behaved in a manner identical to that illustrated in Fig. 2. The material of the first peak was found to be resolved into a normally migrating active component, and an inactive one which still migrated with the column front. It is apparent therefore, that the heterogeneity seen in Fig. 3 is an artifactual result of the conditions of depolymerization and/or dialysis of the protein preparation. The slowest moving, and largest component of the elution diagram shown in Fig. 3 corresponds in its chromatographic behavior to unmodified protein. The two new peaks are held much less tightly onto the column, since they emerge prior to the beginning of the salt gradient. This behavior indicates that the protein emerging in the two fast peaks is more negatively charged than is the unmodified material. The ready restoration of normal chromatographic behavior to these materials by treatment with homocysteine followed by a short dialysis indicates that the modification observed may be due to mixed disulfide formation between the enzyme and thioglycollate in the buffer. In testing this hypothesis, it was observed that the conditions for obtaining appreciable amounts of chromatographic heterogeneity are twofold. In the first place, depolymerization must be effected with concentrations of homocysteine not higher than 3 mg/ml, and secondly, the subsequent dialysis *versus* buffer containing thioglycollate must be for a period of at least 10–12 h. Small variations in protein concentration did not affect these results. When these conditions are met, appreciable amounts of fast moving materials are reproducibly obtained in CM cellulose chromatograms (Fig. 4). The figure also shows that if mercaptoethanol is used in the place of thioglycollate, no fast moving material is obtained, and that the same is true for mercaptoethylamine. With the latter thiol, however, there is a small but significant broadening of the protein band, indicating the presence of a new component which is eluted from CM cellulose columns at a slower rate than is the unmodified protein. The results of these experiments demonstrate that the chromatographic heterogeneity observed is the result of mixed disulfide formation, and further that the mixed disulfides contain the thiol of the buffer and not homocysteine. Mixed disulfides of the protein with the homocysteine used for depolymerization, although probably present, would not be expected to alter the chromatographic mobility of the protein, since at pH 6 there would be essentially no net change in the charge of the protein as a result of such a modification. For similar reasons, one should observe unchanged chromatographic characteristics when mercaptoethanol is employed in the buffer. Mixed disulfides of the protein with thioglycollate would, on the other hand, be expected to result in a more negatively charged protein at pH 6 and therefore account for the presence of fast moving components on CM cellulose columns, while mercaptoethylamine would be expected to have an effect in exactly the opposite direction.

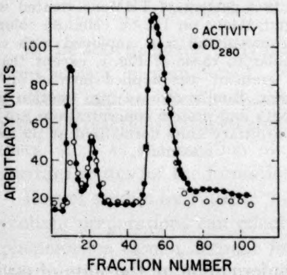

Fig. 3. Chromatography of THMenz on a preparative scale CM cellulose column. The sample size was 250 mg and the column dimensions 2.5 × 15 cm; 19-ml aliquots were collected. The initial solvent was 0.005 M in both phosphate and thioglycollate, and 0.001 M in Versene at pH 6.1. At fraction 35 gradient elution was begun with the initial solvent in the mixing chamber and the same buffer made 0.3 M in sodium chloride in the reservoir. Protein concentration and enzyme activities are expressed in arbitrary units normalized at fraction 54. See the text for discussion of this figure.

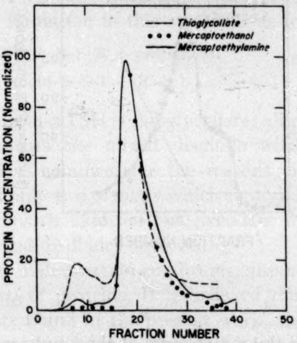

Fig. 4. The superimposed elution patterns of three separate experiments with 1 × 8 cm CM cellulose columns. The patterns shown are normalized and present only protein concentration data. Enzyme activities were found to superimpose well on these curves. 3-ml fractions were collected, under elution conditions identical to those described in Fig. 2 except that the starting buffer was 0.025 M in phosphate and that all solutions were 0.005 M in the appropriate thiol. See the text for further discussion of this figure.

Another kind of mixed disulfide, which can be demonstrated chromatographically, is that formed between THMenz and glutathione. If the enzyme is treated with oxidized glutathione at 37° for 2 h, and then subjected to chromatographic analysis on DEAE cellulose columns, it is found that, in contradistinction to the untreated enzyme, the modified protein is held to the column and eluted as shown in Fig. 5.

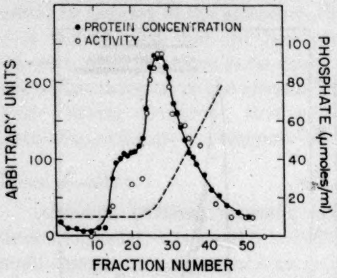

Fig. 5. Elution pattern of THMenz treated with oxidized glutathione on DEAE cellulose column. A 1 × 15 cm column was employed with conditions similar to those of Fig. 1, except that a phosphate gradient was applied instead of the second buffer. Sample size 10 mg, fraction size, 2 ml. Activity and protein concentrations are expressed in arbitrary units normalized at the peak maximum.

DISCUSSION

One of the major aims of this study was to gain information on the state of purity of THMenz. Chromatographic analysis of the enzyme on CM cellulose gives no indication of the presence of any major contaminants and is in agreement with the results presented earlier[1] and in the accompanying paper[4] in suggesting the essential homogeneity of the protein. The chromatographic heterogeneity seen under some conditions of treatment of the protein has been shown to be an artifactual one due to the formation of disulfide bonds between the protein and a thiol of net charge at pH 6. The potentially great sensitivity of chromatographic analysis is again illustrated here since the mixed disulfides observed cannot differ from the unmodified protein by very many charges. However, it is also apparent from the data presented here that alterations of the protein structure which do not affect the net charge may not be reflected in changed chromatographic behavior.

It is perhaps surprising in view of the acidic character of THMenz (isoelectric point of 6.4) that this protein is not held onto DEAE cellulose columns. This fact may be a reflection of an uneven distribution of charges on the surface of the protein so that more cationic groups are available for interaction with the cellulose than anionic ones, even though the latter predominate over the whole molecule. Alternately, it is also possible that the isoelectric point measured is low due to the binding of phosphate ions by the protein under the conditions of the mobility measurements. Such phenomena are known to sometimes cause large variations in the electrophoretic mobility of proteins[7]. DEAE cellulose chromatography has, however, proven to be a useful procedure for the removal of the last major contaminant known to be associated with THMenz, and has been so employed throughout the present work.

It is clear now, as the result of the work of a number of laboratories, that mixed disulfides may readily be isolated from thiol-disulfide systems[8,9]. Indeed, a number of measurements of the equilibrium constants of such systems indicates that they are of the order of 1 and that therefore high concentrations of such products may easily be formed under favorable conditions[10–12]. The results of the present study may be interpreted to mean that the mixed disulfides which were demonstrated on

CM cellulose chromatography are formed as the result of an interchange reaction between the thiol of the buffer and a mixed disulfide of the protein and homocysteine. Such a mechanism is indicated by the fact that the conditions of depolymerization of the polymer by homocysteine determine whether mixed disulfides with thioglycollate may be formed. A highly simplified scheme to illustrate what is believed to be the major pathway of mixed disulfide formation in this system is as follows:

$$\text{Protein–S–S–protein} + \text{RSH} \longrightarrow \text{protein–S–S–R} + \text{protein–SH} \quad (1)$$

$$\text{Protein–S–S–R} + \text{TSH} \longrightarrow \text{protein–S–S–T} + \text{RSH} \quad (2)$$

In the above scheme, RSH is homocysteine and TSH is thioglycollate; all protein species except protein–S–S–T, which represents the mixed disulfide with thioglycollate, migrate identically on CM-cellulose columns, for the reasons outlined above. The reactions outlined above represent only two of many which can take place. Others, such as the reaction of protein–SH with oxidized thioglycollate may be important routes for the formation of the mixed disulfides observed.

Recent studies by WHITE[13] indicate that, under certain conditions, impure thioglycollate preparations can effect a thiolation of proteins. It is believed that such a phenomenon cannot account for the results found in the present work, since the thiolation reaction results in amide bond formation between the thiol esters present in the thioglycollate and the protein. Such a reaction should not be found to be reversible by homocysteine treatment, whereas the mixed disulfides found in the present work are readily cleaved by such treatment. It is therefore felt that thiolation does not represent a major reaction pathway in this system.

The fact that the elution patterns of monomer and polymer preparations of THMenz on CM cellulose are identical is of particular interest in view of the demonstration that small alterations in the charge of the protein are reflected in large changes in chromatographic mobility. It seems apparent that the different forms of the enzyme must be very similar in net charge at pH 6. Thus, if the combination of monomer residues in the polymer molecules involves charged group interaction, this interaction must be such that equal numbers of cationic and anionic side chains are masked.

There is reason to believe that the alterations in chromatographic behavior induced by mixed disulfide formation may be a phenomenon of general significance. Many proteins are known to contain reasonably reactive sulfhydryl groups, and protein mixed disulfides have in the present study been shown to be formed under very mild conditions. During the course of purification, proteins are often exposed to thiols under conditions similar to those used in the present study, and it is not unreasonable to expect that many such protein systems contain appreciable concentrations of mixed disulfides. In view of this consideration, it should be borne in mind that the demonstration, by physical means, of the presence of a number of biologically active components in a protein system does not necessarily indicate that each of the active species represents a different protein. The ease with which a single protein can be induced to exhibit chromatographic heterogeneity as a result of relatively minor structural alterations is at the same time a demonstration of the effectiveness of cellulose column chromatography as an analytical tool and of the potential pitfalls involved when appropriate controls are not carried out or when the system under study is incompletely characterized.

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The author wishes to express his appreciation to Dr. G. L. CANTONI for his very helpful advice and encouragement during the course of this work, and to Mr. H. RICHARDS for his extremely able technical assistance.

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THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

VI. STUDIES OF THE HOMOGENEITY, SHAPE PROPERTIES AND MONOMER FUNCTIONALITY OF THETIN-HOMOCYSTEINE METHYLPHERASE

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SUMMARY

It has been demonstrated, by means of moving partition cell ultracentrifugation, that the sedimentation coefficients of the enzymic activity of thetin–homocysteine methylpherase and of the total protein present in the system are the same over a wide range of degrees of polymerization. This observation is believed to be a highly sensitive criterion of homogeneity, and one which should be applicable to a number of other protein systems which may be studied at different degrees of aggregation.

Hydrodynamic studies of monomer and polymer preparations have shown that the shape of each of the components of THMenz polymer preparations is very close to that of the monomer. Direct observation by electron microscopy has confirmed this result. In accordance with this concept, it has been shown by application of the STOCKMAYER statistical theory of polymerization to the THMenz system that there are three functional groups on each monomer residue which can form intermolecular disulfide bonds in the polymerization reaction and that therefore the polymer molecules are three dimensional aggregates as opposed to linear ones.

INTRODUCTION

Earlier papers in this series have reported on the purification and properties of the enzyme THMenz[1–4], an enzyme which catalyzes the methylation of homocysteine to methionine by dimethylacetothetin. The purified protein behaves as a homogeneous substance in the ultracentrifuge, during free boundary electrophoresis and chromatographically, and is able to undergo an unusual, reversible, depolymerization. Since depolymerization can be effected only with agents which are known to cleave disulfide bonds and since repolymerization of the monomer can be prevented by blocking its sulfhydryl groups with a number of suitable reagents, it has been concluded that

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Abbreviations: THMenz, thetin–homocysteine methylpherase; DEAE, diethylaminoethyl; DPNH, reduced diphosphopyridine nucleotide.

* Twenty-second paper in a series on enzymic mechanisms in transmethylation.

the reversible polymerization is a result of the formation of intermolecular disulfide bonds involving the participation of particularly reactive sulfhydryl groups.

It has been estimated that the purified THMenz represents about 1% of the protein of horse liver; its preferred substrate, dimethylacetothetin, is not known to occur in nature, and therefore there is some uncertainty as to the physiological role of this protein. Moreover, while THMenz can utilize betaine as a methyl donor at a slow rate, a more specific betaine homocysteine methylpherase has recently been purified in this laboratory from horse liver[5]. It became important, therefore, to obtain further information on the state of purity of THMenz in order to be able to design studies which might provide evidence on the physiological role of this interesting protein. A detailed comparison of the properties of the enzyme in its monomeric and polymerized states has also been carried out in order to gain further insight into the mechanism and significance of the polymerization process.

The results of the experiments reported in this paper confirm fully the earlier findings and provide strong evidence that THMenz is, within reasonable limits, a homogeneous protein and that both the polymerization and enzymic activity are properties of the same molecular species. Furthermore, it has been possible to deduce that three of the functional groups (presumably sulfhydryl) of each molecule are capable of taking part in the polymerization reaction to produce a highly cross-linked and symmetrical polymer molecule whose shape is very similar to that of the closely spherical monomer.

MATERIAL AND METHODS

THMenz was prepared as described previously[1] with the modifications introduced in a more recent publication from this laboratory[2]. The enzyme preparation was carried through the ammonium sulfate step, and then further purified with DEAE cellulose columns as described in the accompanying paper[4].

Protein concentrations were determined routinely from absorption measurements at 280 mμ measured in a Beckman DU spectrophotometer. The extinction coefficient used was 13.0 for a solution containing 1 g of protein in 100 ml of solution. This value was obtained from dry weight measurements and was found to be essentially independent of the degree of polymerization of the protein.

Viscosity measurements were performed with a capillary viscometer in a water bath at $20° \pm 0.02°$ with an outflow time of 103 sec for water. Sedimentation experiments were performed in a Spinco Model E Analytical Ultracentrifuge, and all sedimentation coefficients were converted to water at 20°, assuming a partial specific volume of 0.75 for the protein[2]. Area measurements were made from tracings of enlarged schlieren patterns. Overlapping peaks were resolved into individual gaussian components (see ref. 6). All physical measurements were performed with the protein dissolved in 0.05 *M* potassium phosphate buffer, pH 7.4, unless otherwise specified.

Enzyme activity measurements were performed as previously described, with dimethylacetothetin serving as methyl donor[1].

RESULTS

Partition cell experiments

The development of the theory of the moving partition ultracentrifuge cell by YPHANTIS AND WAUGH[7], along with the commercial availability of the apparatus which they designed, has provided a powerful tool for the study of substances of biological interest. This technique has been applied in the present work in order to decide whether the enzymic activity of THMenz is a property of the same molecular species as is the reversible polymerization, and also as an unusually sensitive criterion of purity.

Solutions of THMenz of varying degrees of polymerization, at protein concentrations in the range of 0.1–1 g/100 ml, were sedimented in the partition cell, at either 44,700 rev./min or 59,780 rev./min, for varying periods of time, so that somewhere between 25 % and 75 % of the total material had moved below the separation plate at the end of the experiment. Protein concentrations as well as enzymic activities were determined in both the top and the bottom compartments of the cell, as well as in the original solution, and weight average sedimentation coefficients were calculated for both the enzymically active species and the total protein[7]. It is important in using the partition cell that essentially quantitative recoveries of material are obtained, and all of the data reported satisfy this condition. It had been observed in some early experiments that erratic results may be obtained if the recoveries are appreciably less than 95 %. In order to insure against inactivation of the enzyme during the ultracentrifugal run, most experiments were performed at 5–10° in the presence of 0.005 *M* Versene.

The results obtained from these experiments are presented in Fig. 1. In the figure, the sedimentation coefficients obtained from activity measurements are plotted against those derived from measurements of total protein concentration. The line, with a slope of one and intersecting the origin represents the expected distribution of the data if the protein were indeed homogeneous. The experimental points conform quite closely to the expected distribution. For the sedimentation coefficients of the activity and of the protein to be the same by chance, at all degrees of polymerization, is an extremely unlikely occurrence. Such agreement must be interpreted as providing strong evidence for the identity of the enzymically active protein with the total protein present, and thus for the homogeneity of the preparation under consideration. The calculated sedimentation coefficients are sensitive to small errors in the concen-

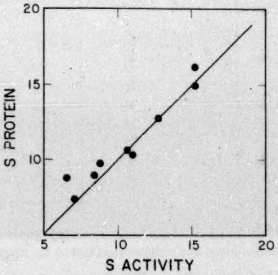

Fig. 1. Sedimentation coefficient ($s_{20,w}$) of THMenz activity as a function of the sedimentation coefficient of the total protein, for preparations of varying degrees of polymerization. ———, the expected distribution of the data for a homogeneous protein. The samples studied vary from monomer (S = 8) to preparations with a weight average degree of polymerization of 3 (S = 16).

tration measurements, and in the present work, it is conceivable that impurities present at a level of 5–10 % on a weight basis might not be detected.

Shape properties

In an earlier paper in this series[1], it was suggested, on the basis of ultracentrifugal measurements made at a single protein concentration, that the sedimentation coefficients of the various components of the polymer are in very close agreement with those calculated on the assumption that no shape or density change occurs during polymerization. Since these calculations carry important implications as to the geometry and configuration of the molecular species present in the polymeric system, it was considered of interest to study the concentration dependence of the sedimentation coefficients of the polymer, as well as of the isolated monomer. The results of these experiments are shown in Fig. 2. It is apparent that the rates of sedimentation of the monomer, dimer, trimer and tetramer are all independent of concentration in the range studied. This result is thought to be consistent with the highly symmetrical nature of the species in question[3]. It is also of interest to note that the sedimentation coefficient of the isolated monomer in the presence or the absence of the other components of the polymeric mixture is identical.

The assumption that no shape change occurs during the polymerization of the monomer was tested further by making viscosity measurements of a polymer preparation with a weight average sedimentation coefficient of 15 S and the monomer (S = 8). In Fig. 3 the specific viscosities of the monomer (open circles) and of the polymer (closed circles) are presented as a function of protein concentration. It is apparent that there is little, if any, change in viscosity accompanying the interconversion of monomer and polymer. These data support the concept that the shape of the polymeric components is indeed similar to that of the monomer. Furthermore, the low intrinsic viscosity observed here agrees with calculations based on the diffusion of the monomer[3] in suggesting that the THMenz molecule in solution is close to spherical in shape.

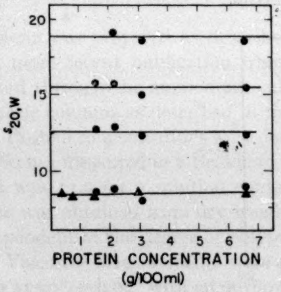

Fig. 2. Concentration dependence of the sedimentation coefficients of some of the components of THMenz polymer. The lines represent, reading from bottom to top, monomer, dimer, trimer, and tetramer. The triangles are data obtained with isolated monomer, while the other points were obtained by analysis of sedimentation patterns of polymeric mixtures.

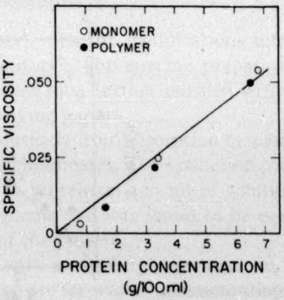

Fig. 3. ○—○, specific viscosity of THMenz monomer; ●—●, specific viscosity of THMenz polymer as a function of protein concentration.

A more direct confirmation of the hypothesis under investigation was sought

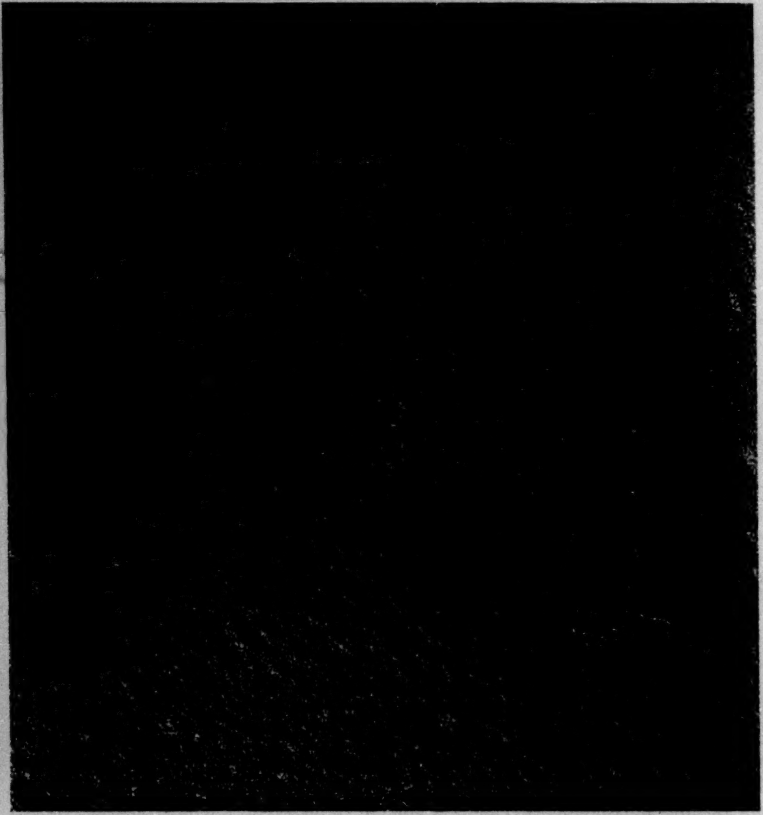

Fig. 4. Electron micrograph of a carbon replica of palladium shadowed THMenz polymer.

by means of electron microscopy. Electron micrographs of a highly polymerized preparation of THMenz were kindly prepared by Dr. L. W. LABAW of these Institutes. An approximately 0.005-% solution of the protein was dried on a colloidion film, shadowed with palladium and then coated with carbon. A typical field of such a carbon replica is shown in Fig. 4. Because of the similarity in size of the monomer units and the grains representing the substrate background no useful conclusions may at present be drawn about the size and shape of the smaller units by means of this technique. It is apparent, however, that there is a large range of particle sizes, some of which particles correspond to very large molecular aggregates (of the order of 50–100 monomer residues). It may be noted that, as a first approximation, the shapes of all of the particles are the same and that they are also quite symmetrical. These observations are in good agreement with the results of the hydrodynamic measurements discussed above.

Monomer functionality

It has been observed that a concentrated solution of THMenz will gel on standing at 2° for a period of a few days. This behavior is interpreted to mean that at least three functional groups exist, per monomer unit, which are capable of forming intermolecular bonds[8]. A bifunctional monomer could give rise only to straight chain polymers which would not be expected to form gels.

This observation prompted an investigation of the distribution of the various species in preparations of varying degrees of polymerization in order to obtain more reliable data on monomer functionality. The statistical theory of STOCKMAYER[9] may be expressed as follows[10]:

$$Wn = \frac{(fn - n)!\, f}{(n - 1)!\, (fn - 2n + 2)!}\, p^{n - 1} (1 - p)^{(fn - 2n + 2)}$$

where W_n is the weight fraction of n-mer, f is the functionality of the monomer and p represents the fraction of the functional groups which have reacted. This relationship is valid only if intramolecular reactions do not occur and if, at any stage, of the polymerization, all unreacted functional groups have an equal chance of reacting, no matter what size molecule they are attached to. The STOCKMAYER theory predicts that, for systems in which there are three or more functional groups on the monomer, no matter what the degree of polymerization, there will always be more n-mer than $(n + 1)$ – mer. On the other hand, if only two functional groups are present on the monomer, then at some degrees of polymerization the distribution of molecular species will be such that there will be less monomer than dimer. Since out of more than 50 polymer preparations, of varying degrees of polymerization, which have so far been examined in the ultracentrifuge none was found to have more dimer than monomer, it seemed likely on these grounds also that the monomer must carry at least 3 functional groups.

A more quantitative application of the STOCKMAYER theory was attempted after estimation of the weight fraction of monomer, dimer, trimer and tetramer from area measurements made on ultracentrifugal patterns of THMenz preparations of varying degrees of polymerization. These experiments were carried out in double sector ultracentrifuge cells containing solvent in one sector in order to obtain accurate base lines for the area measurements. The total amount of material present in a given cell was estimated, in area units, from schlieren patterns obtained at very early times during the run when the boundary had completely separated from the meniscus, but a plateau region was still present. All area measurements were corrected for radial dilution, but no attempt was made to apply JOHNSTON-OGSTEN corrections. As shown above, the rate of sedimentation of each of the species of interest is independent of concentration and therefore JOHNSTON-OGSTEN anomalies are not an important consideration in this system[11,12]. From such area measurements one obtains directly the value of W_1, W_2, W_3, and W_4. By substituting the value of W_1 so determined into the equation given above, and assuming a value for f of either 2, 3, or 4, one obtains the value of p which corresponds to the chosen functionality. Employing these corresponding values of p and f, one may calculate the theoretical distribution of components for each of the three functionalities chosen. A comparison of the measured distribution with those calculated, Fig. 5, has, in all cases, shown that the functionality of the monomer more closely approximates three than four, and cannot be as low as two. It is apparent, however, that the experimental points deviate from the theoretical curve. Perhaps the most likely cause of this anomalous behavior is that a small percentage of the functional groups of the monomer are not free, but have been converted to mixed disulfides during the depolymerization process. That such a situation actually exists is not unlikely in view of the chromatographic evidence for mixed disulfide formation discussed in the accompanying paper[4].

It should be pointed out, however, that there is much more uncertainty about the area measurements of trimer and tetramer than those of monomer and dimer. This is due to the fact that the latter two components are present in greater amounts and are more completely separated from each other in the schlieren patterns. Thus, a part of the observed deviation may be an apparent one.

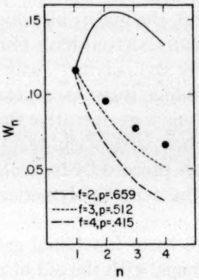

Fig. 5. The weight fraction of monomer, dimer, trimer and tetramer in a typical sample of THMenz polymer. The lines are theoretical distributions calculated as indicated in the text, for assumed functionalities of ———, 2; ······, 3; – – – –, 4. The corresponding values of p employed were 0.659, 0.512, and 0.415 respectively.

DISCUSSION

The happy chance that THMenz can undergo a reversible polymerization reaction and also has an enzymic activity which is stable to changes in its state of aggregation enables the application of partition cell centrifugation as an unusually sensitive method of studying the homogeneity of this protein. The demonstration that the sedimentation coefficients of both the total protein present and of the enzymic activity are the same over a wide range of polymerization represents strong evidence for the homogeneity of THMenz. This conclusion is supported by the electrophoretic and sedimentation measurements which have been reported by DURELL *et al.*[1], and also by the behavior of the enzyme on CM columns[4]. It is apparent that any macromolecular system which can be studied at different degrees of aggregation or unfolding and for which two different properties of the molecule of interest can be independently measured is amenable to partition cell analysis as a criterion of homogeneity. Absolute purity is, however, a state which can only be approached in an asymptotic manner. There are indications that THMenz is not completely free of contaminating proteins. Thus, it has been found that THMenz preparations have DPNH oxidase activity, and it has been established, with the partition cell, that the THMenz and DPNH oxidase activities are functions of different protein species. It has been possible to calculate that the DPNH oxidase must be present in very small amounts in the

THMenz preparations, amounts which are outside the limits of detectability of the partition cell experiments described in this paper.

Perhaps the most rigorous criterion of homogeneity available in protein chemistry is that of constant solubility[13]. Attempts to apply this test to THMenz have not been successful, but it may be pointed out that the monomer undergoes partial polymerization during the prolonged equilibration periods required in solubility determinations. Thus, the number of components of such a system does not remain constant with time, and therefore the phase rule criterion of purity cannot be applied.

Throughout this paper it has been tacitly assumed that the mechanism of the polymerization process is one involving the oxidation of two sulfhydryl groups, each on a different molecule, to produce an intermolecular disulfide bond. Although there is a good deal of evidence which indicates that the primary bonds formed during the polymerization are disulfides[1-3], the means by which these intermolecular bonds form are more obscure. DURELL AND CANTONI have found a small difference between the sulfhydryl contents of monomer and polymer[2], which would indicate an oxidative mechanism, but some type of disulfide interchange reaction has not yet been ruled out. If a mechanism of the latter type were operative then the three functional groups would not be sulfhydryls, but rather particularly reactive disulfide groups. It is not inconceivable that the reaction may proceed by both of these mechanisms simultaneously. More detailed studies of the actual polymerization process are required to answer this question.

The conclusion that there are three functional groups on a monomer unit has been arrived at in an indirect manner, with the aid of reasonable, but only partially tested assumptions. Although the conditions which obtain during the polymerization of a number of small organic molecules have been shown to comply, to a first approximation, with the requirements of the STOCKMAYER theory[14], there have been no comparable studies with protein systems.

The physiological rôle of THMenz has long been of interest to this laboratory[1]. Since the enzyme has been shown to be essentially homogeneous, the calculation that it represents about 1 % of the total horse-liver proteins is strengthened. In view of the fact that a separate enzyme has recently been isolated from horse-liver preparations which also catalyzes the methylation of homocysteine to form methionine[5], and which accounts for about 50 % of the betaine-homocysteine methylpherase activity of horse-liver extracts, the large amounts of THMenz present in this tissue seems surprising. It is possible that the primary physiological role of this protein is related more closely to the reversible polymerization which it can undergo than to its enzymic activity. It is particularly interesting in this regard to note that the THMenz isolated from rat liver by FROMM AND NORDLIE[15] exhibits ultracentrifugal behavior which is analogous to that of the horse-liver enzyme[5]. The work of MAZIA and others[16] indicates that the protein of the mitotic apparatus may be capable of a reversible polymerization with the formation of intermolecular disulfide bonds, and that this process may play an important role in mitosis. A similar system may also be involved in the cell wall protein of yeast during the budding process[17].

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SEPARATION AND PROPERTIES OF DPN AND TPN-LINKED SUCCINIC SEMIALDEHYDE DEHYDROGENASES FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY

Pseudomonas aeruginosa oxidizes succinic semialdehyde to succinate.

From a soluble extract of the cells, DPN and TPN-linked succinic semi aldehyde dehydrogenases were separated, partially purified, and their properties described.

INTRODUCTION

Recently JAKOBY AND SCOTT[1] reported that the partially purified SSA dehydrogenase from *Pseudomonas fluorescens* requires pyridine nucleotide and that TPN was 8.2 times more active than DPN. The present studies with *Ps. aeruginosa* show that there are two different SSA dehydrogenases, one DPN and one TPN-linked. The separation, purification and properties of both enzymes are described.

MATERIALS AND ANALYTICAL METHODS

Materials

The following chemicals were obtained from commercial sources: DPN, TPN (Sigma, Pabst), DPNH, TPNH, ATP, ADP, AMP, and CoA (Sigma) and EGTA was kindly supplied by Dr. C. N. REILLEY. SSA was obtained by the hydrolysis of formyl succinic acid diethylester before every experiment by the modified method of BESSMAN *et al.*[2]. Formyl succinic acid diethylester was synthesized by the method of CARRIERE[3]. Malonic semialdehyde was synthesized by the hydrolysis of potassium β-diethoxy propionate by the modified method of BESSMAN *et al.*[2] for SSA. Potassium β-diethoxy propionate was synthesized by the method of WOHL AND EMMERICH[4]. Calcium phosphate gel, aged at least two months before using, was prepared by the method of KEILIN AND HARTREE[5].

Abbreviations: SSA, succinic semialdehyde; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; CoA, coenzyme A; EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N′-tetraacetic acid; GABA, γ-amino butyric acid; EDTA, ethylene diamine tetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane chloride. PCMB, *p*-chloromercuribenzoate.

Biochim. Biophys. Acta, 45 (1960) 554–560

Analytical determinations

Protein was estimated by the method of LOWRY *et al.*[6] and the method of WARBURG AND CHRISTIAN[7] was used for the enzyme after the protamine treatment. SSA was determined by the method of BESSMAN *et al.*[2] and chromatographically by using the solvent system of FLAVIN AND OCHOA[8]. MICHAELIS constants were determined according to the method of LINEWEAVER AND BURK[9]. Acetyl and succinyl phosphate were determined by the hydroxamate method of LIPMAN AND BURK[10]. DPN and TPN were assayed by the method of COLOWICK *et al.*[11].

EXPERIMENTAL

Preparation of the enzymes

Ps. aeruginosa utilized GABA as a sole source of carbon and nitrogen. From a growing culture, SSA and succinate were identified by chromatography using the solvent system of FLAVIN AND OCHOA[8]. The cells also utilized arginine, putrescine and glutamate as a sole source of carbon and nitrogen; and β-alanine and malonate as a source of carbon. Ethylene diamine, propylene diamine, cadaverine, acetate, propionate, asparatate and ornithine were not utilized for growth.

The cells were grown aerobically in the following medium: dibasic potassium phosphate 0.15 %; monobasic potassium phosphate 0.05 %; $MgSO_4$ 0.01 %; GABA 0.5 % and yeast extract 0.1 %. GABA was sterilized by filtration through a Hercules Filter L-3 and added to the previously autoclaved inorganic medium. Cultures were harvested after 24 to 48 h with shaking at 37°, the cells were separated by centrifugation and washed twice with 0.01 *M* potassium phosphate buffer pH 7.2 containing 5 m*M* of mercaptoethanol.

Enzymic activity was measured by observing the rate of DPNH and TPNH formation spectrophotometrically at 340 mμ and the molecular extinction coefficient of $6.22 \cdot 10^{-6}$ cm^2/mole for DPNH and TPNH was employed[12]. The 0.5-ml reaction mixture for the measurement of activity contained SSA 0.2 μM, TPN or DPN 0.5 μM, $MgCl_2$ 0.5 μM, tris-(hydroxymethyl)aminomethane chloride at pH 8.7, 20 μM and the enzyme which contained about 0.5 μM of mercaptoethanol. The reaction was followed at 25° after the addition of 0.2 μM of SSA. A unit of enzyme activity was defined as the amount catalyzing the formation of 1 mμM of DPNH or TPNH per minute in this standard reaction mixture. Specific activity was defined as the number of units of activity/mg enzyme protein.

Unless otherwise noted, all buffers used for the preparation were 0.01 *M* of potassium phosphate buffer pH 7.2 which contained 5 m*M* of mercaptoethanol. Centrifugations were carried out at 14,000 × *g* for 30 min and all procedures were conducted in a cold room at approximately 4°. Suspensions of washed cells which contained 1 g of wet weight/10 ml of buffer, were sonicated in a 9-kc Raytheon oscillator under cooling for 10 min. The cellular debris was removed by centrifugation at 14,000 × *g* for 1 h. The supernatant solution usually contained a small amount of pyridine nucleotide oxidases, which interfered with the determination of SSA dehydrogenases. 50 mg of protamine sulfate (2 % solution) was then added per gram of protein. The precipitate collected by centrifugation was discarded and the supernatant was dialyzed against buffer for 15 h.

TPN-linked enzyme

To 118 ml of the protamine supernatant, 17 g solid ammonium sulfate was added (0.225 saturation). The precipitate was removed by centrifugation and discarded. A precipitate was formed by the further addition of 17 g of ammonium sulfate to the supernatant solution (0.225 to 0.450 saturation). This precipitate was centrifuged and dissolved in 82 ml of buffer and refractionated by the addition of 13 g of solid ammonium sulfate. The small precipitate obtained was removed by centrifugation. To the clear supernatant 13 g of ammonium sulfate was added. This precipitate was centrifuged, redissolved in 70 ml of buffer and dialyzed 15 h against buffer.

To 70 ml of this preparation, 2 mg (dry wt.) of calcium phosphate gel/mg of protein was added. The gel was evenly suspended and gently stirred for 30 min and then centrifuged at 7,000 × *g*. If enzyme activity still remained in the supernatant, more calcium phosphate gel was added to the solution. The combined gel fractions containing the enzyme were eluted twice with 20 ml of 0.1 M of potassium phosphate buffer pH 7.2 and the eluate was dialyzed against buffer for 15 h. To 40 ml of the gel eluate 6.3 g of ammonium sulfate was added and the small precipitate was removed by centrifugation. The precipitate obtained by the further addition of another 6.3 g of ammonium sulfate was dissolved in 40 ml of buffer and dialyzed 15 h against buffer.

This fraction was then dialyzed against buffer containing $1 \cdot 10^{-3}$ M of EDTA for 15 h and then dialyzed against EDTA-free buffer for another 15 h. Twelve ml of this preparation contained 18 mg of protein, and had 273 units of total activity or 15.3 of specific activity. TPN was about 19 times more active than DPN. It was not possible to get a preparation completely free of the DPN enzyme.

DPN-linked enzyme

To the 119 ml of the supernatant obtained after the precipitation of TPN enzyme (step 3), 24.8 g of ammonium sulfate was added (0.450 to 0.750 saturation). The precipitate was collected by centrifugation and dissolved in 70 ml of buffer. The solution was refractionated by the addition of ammonium sulfate to collect the precipitate formed between 0.450 and 0.750 saturation and this was dissolved in 44 ml of the buffer, and dialyzed 15 h against buffer. 207 mg of the gel (dry wt.) was added to 44 ml of this fraction. The suspension was gently stirred for 30 min and then centrifuged. 37 ml of the supernatant was dialyzed 15 h against buffer. To 37 ml of the gel supernatant, 11.7 g of ammonium sulfate was added and the resultant precipitate was removed by centrifugation. To the supernatant obtained 7.8 g of ammonium sulfate was added. The precipitate formed was collected by centrifugation, dissolved in 12 ml of buffer and dialyzed 15 h against buffer containing $1 \cdot 10^{-3}$ M of EDTA and then against EDTA-free buffer for 15 h. It was then immersed in a waterbath at 50° for 10 min. The heated preparation was cooled to —20° immediately after the heating. After thawing, the cloudy precipitate was removed by centrifugation. The clear supernatant contained only the DPN-linked enzyme. Table I summarizes these procedures.

Properties of the enzymes

Specificity: Both the purified enzymes had no activity with formaldehyde, acetaldehyde, propionaldehyde, isovaleraldehyde, benzaldehyde, anisaldehyde, glyceraldehyde and glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate dehydro-

TABLE I

PURIFICATION OF TWO SUCCINIC SEMIALDEHYDE DEHYDROGENASES FROM *Pseudomonas aeruginosa*

The reaction mixture for DPN-linked enzyme system contained 0.2 μM of SSA, 0.5 μM of DPN, 0.5 μM of $MgCl_2$, 20 μM of Tris buffer pH 8.7. For TPN-linked enzyme system it contained 0.2 μM of SSA, 0.5 μM of TPN, 1.0 μM of $MgCl_2$, 20 μM of Tris buffer pH 8.6. Final volume 0.5 ml; Temperature, 25.5°.

Step	*Procedure*	*Total volume (mg)*	*Total protein (mg)*	*Total activity units*	*Specific activity units/mg*	*Activity ratio TPN/DPN*
	TPN-linked enzyme					
1	Crude extract	110	1,097	5,450	4.96	0.74
2	Protamine	118	542	6,020	11.1	0.51
3	1st Ammonium sulfate	82	288	2,250	7,81	4.9
4	2nd Ammonium sulfate	70	173	1,700	9.83	7.1
5	Calcium phosphate gel eluate	41	52	172	3.31	8.4
6	3rd Ammonium sulfate	13	25	208	8.25	12.0
7	EDTA treatment	12	18	273	15.3	19.0
	DPN-linked enzyme					
1	Crude extract	110	1,097	7,320	6.67	1.3
2	Protamine	118	542	11,850	20.93	2.0
3	1st Ammonium sulfate	70	252	7,650	30.18	3.5
4	2nd Ammonium sulfate	44	207	6,400	31.90	4.2
5	Calcium phosphate gel negative	37	69	674	9.64	9.4
6	3rd Ammonium sulfate	12	27	560	20,37	13
7	EDTA treatment	11	22	1,010	46.70	35
8	Heat treatment	10	9.4	744	78.80	∞

genase was reported to oxidize SSA by HARTING AND VELICK[13]. However, the TPN-linked enzyme had activity with malonic semialdehyde, though SSA was 3.5 times more active. The DPN-linked enzyme did not oxidize malonic semialdehyde, and could not use TPN. The TPN-linked enzyme still reduced DPN, though its activity was only 5–15 % of that of TPN.

Stability: Both the crude extracts, after storage at —20° for a week, showed about 80 % loss of their original activities. After purification, the loss was 15 to 20 % in a month. Throughout the purification procedure and storage, the presence of 5 mM of mercaptoethanol was essential for stability. The enzymes were rapidly inactivated at pH values below 6.5 and above 8.5.

Effect of pH: The DPN-linked enzyme had a sharp pH optimum at about pH 8.7, which was independent of the three buffers used. The maximum velocity was greatest in Tris buffer, less in bicarbonate and veronal. The TPN-linked enzyme, had an optimum at about pH 8.6 in veronal, Tris and bicarbonate buffers. The velocities in the last two buffers were lower than in veronal. The pH curves are given in Fig. 1. Potassium phosphate buffer inhibited both enzymes.

Stoichiometry: With 4 ml of the reaction mixture which contained 2.0 μmoles SSA, 4 μmoles DPN, 4 μmoles $MgCl_2$, 80 μmoles Tris buffer pH 8.7 and 740 μg of DPN-linked enzyme (step 8, activation ratio ∞), 100 μmoles of SSA were oxidized for each 115 mμmoles of DPNH formed in the first 2 min and 142 mμmoles of SSA were oxidized per 150 mμmoles of DPNH formed in 5 min. Thus for each mole of DPNH formed, one mole of SSA was oxidized.

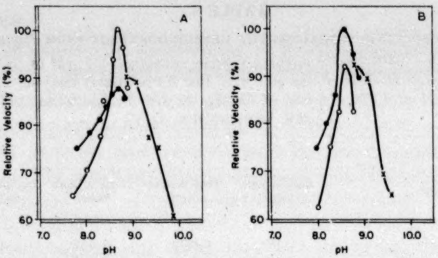

Fig. 1. Effect of pH on the initial velocities of DPN and TPN-linked enzymes. The reaction mixture for the DPN-linked enzyme system contained 0.2 μM of SSA, 0.5 μM of DPN, 0.5 μM of $MgCl_2$, 20 μM of buffer and 140 μg of enzyme (step 8). Final volume, 0.5 ml; temperature, 25.5°. For the TPN-linked enzyme system, the components are the same as in the DPN system, except of TPN, 1.0 μM of $MgCl_2$ and 220 μg of TPN enzyme (step 7; activity ratio 19.0). ○—○, Tris buffer; ●—●, veronal buffer; ×—×, carbonate buffer. A, DPN-linked enzyme; B, TPN-linked enzyme.

Effect of enzyme concentration: For the DPN-linked enzyme, the initial velocity was directly proportional between 50 and 150 μg of protein and for the TPN-linked enzyme between 100 and 200 μg of protein.

Effect of substrate concentration: MICHAELIS constants for SSA and DPN or TPN were determined over SSA concentrations ranging from $5 \cdot 10^{-5}\,M$ to $4 \cdot 10^{-4}\,M$ and pyridine nucleotide concentrations from $3 \cdot 10^{-4}\,M$ to $8 \cdot 10^{-3}\,M$. The results are shown in Fig. 2.

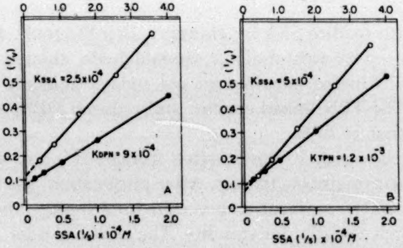

Fig. 2. The effects of succinic semialdehyde and DPN or TPN concentrations on the initial velocity. The reaction mixtures were the same as given in Table I.

Effect of metal ions: $MgCl_2$ increased the reaction velocities of both enzymes. $1 \cdot 10^{-3}$ and $2 \cdot 10^{-3}\,M$ of $MgCl_2$ resulted in 148 and 150% increases in the initial velocities of the DPN-linked enzyme system, and 69 and 143% increases in that of the TPN-linked enzyme system. As shown in Fig. 3, for each initial concentration of DPN a maximal velocity was reached when the $MgCl_2$ concentration was equal to that of DPN, and for the TPN enzyme a maximal velocity was reached when the $MgCl_2$ concentration was twice that of TPN. Without $MgCl_2$ the activity was 43% of that of the complete system for the DPN-linked enzyme and 41% for the TPN-linked enzyme. This difference in the molecular ratio of $MgCl_2$ to pyridine nucleotides suggests that the Mg-pyridine nucleotide complexes are substrates for the SSA dehydrogenases.

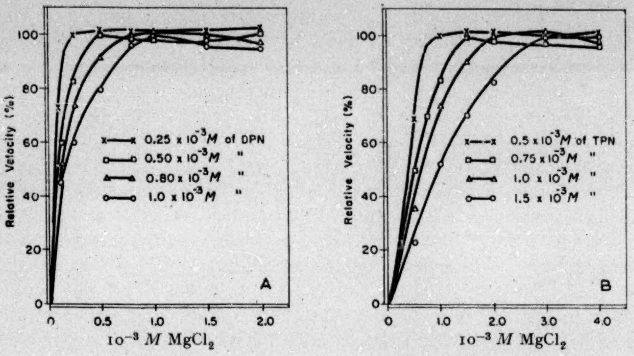

Fig. 3. The effect of $MgCl_2$ on the initial velocity in the presence of various concentrations of DPN or TPN. The reaction mixtures were the same as given in Table I except for $MgCl_2$.

Similar molecular complexes have been proposed by KUBY *et al.*[14] for the relation of ATP and $MgCl_2$ in ATP-creatine transphosphorylase.

Both calcium and manganese increased the rate, but to a lesser extent than magnesium. Other divalent ions were inactive or inhibitory (the pH was lowered to 7.2 to prevent precipitation). Inhibition by EDTA, which occurred in the earlier steps of purification and in the absence of $MgCl_2$, was partially overcome by the addition of $MgCl_2$, $CaCl_2$ or $MnCl_2$ in this order, as shown in Fig. 4. However, there was no difference in the recovery rates from the EGTA inhibition with $MgCl_2$ or $CaCl_2$, although SCHMIDT AND REILLEY[15] showed that the stability-constant of EGTA-Ca was 10.7 and that of EGTA-Mg 5.4. 8-hydroxy quinoline and *O*-phenanthroline each at $1 \cdot 10^{-3}$ M showed 80 and 30 % respectively. There was no quantitative or qualitative difference between DPN and TPN-linked enzymes in their reactions to the addition of the other metal ions or various inhibitors.

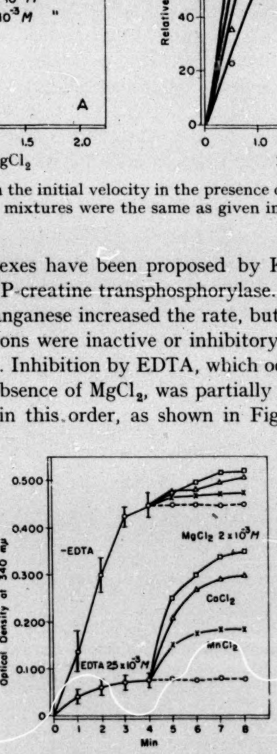

Fig. 4. The effect of $MgCl_2$, $CaCl_2$ or $MnCl_2$ on the inhibition by EDTA. The reaction mixture contained 0.2 μM SSA, 0.5 μM DPN, 20 μM Tris buffer pH 7.2 and 270 μg DPN-linked enzyme of step 5 (activity ratio 9.9). Addition of the metal ion after 4 min.

Effect of sulfhydryl compounds: During the purification procedures of both enzymes, there were great losses in enzyme activities unless mercaptoethanol was added. *p*-chloromercuribenzoate, added in a final concentration of $1 \cdot 10^{-5}\,M$ to the enzymes containing about $1 \cdot 10^{-3}\,M$ of mercaptoethanol, caused an inhibition of 93 %. The addition of glutathione, $1 \cdot 10^{-3}\,M$, reactivated them. But when the enzymes were preincubated with SSA, the same concentration of PCMB showed no inhibition. Mercuric chloride and cupric chloride both at $1 \cdot 10^{-4}\,M$ resulted in 80 to 90 % inhibition. Sodium arsenite, which was reported by JAKOBY[16] as a selective reagent for free sulfhydryl groups, was not as effective as the heavy metals or PCMB. These results indicate that the sulfhydryl group is one of the essential components in the enzyme protein for the oxidation of SSA to succinate.

Effect of ATP or CoA: No stimulatory effects were found upon the addition of ATP up to $1 \cdot 10^{-2}\,M$. With or without ATP, CoA was not required even after the treatment of both enzymes with Dowex-1 resin for the removal of any CoA by the method of STADTMAN *et al.*[17]. There was no evidence for the formation of succinyl CoA, malonyl CoA, acetyl CoA, succinyl phosphate, malonyl phosphate and acetyl phosphate.

Reversibility. In the presence of succinate neither DPNH nor TPNH was oxidized in the presence or absence of $1 \cdot 10^{-3}\,M$ of semicarbazide, added to trap the semialdehyde which might be formed. Semicarbazide in this concentration did not inhibit the reactions.

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OXIDATIVE PHOSPHORYLATION AND RESPIRATORY CONTROL IN HOUSEFLY MITOCHONDRIA*

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SUMMARY

Mitochondria isolated from housefly thoraces were found to resemble other animal mitochondria in their ability to oxidize pyruvate rapidly, in contrast to the reports of other investigators. High P/O ratios were also obtained without addition of serum albumin to the incubation medium and it was shown, by two procedures, that the rate of respiration in these preparations is very markedly controlled by the availability of a phosphate acceptor.

INTRODUCTION

Studies of oxidative phosphorylation and related processes in insect mitochondria were made possible in recent years by the pioneering experiments of LEWIS AND SLATER[1] and of SACKTOR[2]. Prior to this time investigations made on intact insects had revealed that certain flying insects, such as bees and flies, required an extremely rapid expenditure of energy to sustain flight. In such insects the rate of oxygen consumption, upon initiation of flight, was estimated to be 50–100 times the rate of respiration at rest[3].

Early studies by WATANABE AND WILLIAMS[4], by SACKTOR[2], and by HOSKINS, CHELDELIN AND NEWBURGH[5] had indicated the presence of the citric acid cycle in thoracic mitochondria from houseflies and from honey bees, suggesting that the reactions associated with this cycle might be of primary importance in supplying the energy for flight. However, glycolysis in insect muscle produces equimolar quantities of pyruvate and α-glycerophosphate[6,7] and flight muscle is unique in its high content of α-glycerophosphate dehydrogenase activity[8]. In recent manometric studies with mitochondria from housefly thoraces SACKTOR AND COCHRAN[9] found that, when short

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Abbreviations: ADP and ATP, adenosine di- and tri-phosphate, respectively; CoA, coenzyme A; DPN and TPN, di- and tri-phosphopyridine nucleotide, respectively; EDTA, ethylenediamine tetraacetate; P/O, μmoles of phosphate esterified per μg atom of oxygen consumed; Q_{O_2}, microliters of oxygen consumed/h/mg of mitochondrial protein; TPP, thiamine pyrophosphate; Tris, tris(hydroxymethyl)aminomethane.

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equilibration times were used, the rate of α-glycerophosphate oxidation was approximately twice that of succinate, glutamate, or α-ketoglutarate. When measurements were made over very short time intervals (up to 3 min) using the vibrating platinum electrode, CHANCE AND SACKTOR[10] found that the rate of α-glycerophosphate oxidation was ten times the rate of succinate oxidation and 50 times the rate for pyruvate. Under these conditions they obtained a Q_{O_2} of 215 for the oxidation of α-glycerophosphate in a phosphorylation medium, which would suggest a Q_{O_2} of only 4 or 5 for pyruvate oxidation. CHANCE AND SACKTOR[10] suggested, therefore, that α-glycerophosphate is the principal substrate for the activation of the respiratory chain during flight and that the citric acid cycle is not of primary importance in flight metabolism.

CHANCE, SACKTOR, COCHRAN AND KING[9-11] also found that in their insect mitochondrial preparations the rate of respiration was independent of the concentration or presence of ADP, in contrast to the situation commonly encountered in other animal tissues. This led to the proposals that the respiratory rate in these flight muscles is controlled by the concentration of α-glycerophosphate[10] or the activity of the α-glycerophosphate oxidase[12]. More recently BUCHER, KLINGENBERG AND ZEBE[13] demonstrated respiratory control, by the ADP concentration, in mitochondria from locust muscle. Earlier GONDA, TRAUB AND AVI-DOR[14] had isolated mitochondria from whole mosquitoes which showed respiratory control by the ADP concentration, though with rather low acceptor ratios. SACKTOR[15] reported similar effects with minces of housefly muscle. However, control of respiration by the ADP concentration has not been demonstrated previously in well-washed mitochondria from flies.

Our investigations have been made with housefly mitochondria isolated by a somewhat different technique than used by others. These mitochondria oxidize pyruvate at much higher rates than those reported by CHANCE AND SACKTOR[10], and require the presence of a phosphate acceptor for maximal respiration.

METHODS AND MATERIALS

Mitochondria were isolated as described previously[16], except that in some experiments only the thoraces of the adult houseflies (*Musca domestica*) were used as source material. In the procedure described previously a mortar and pestle was used for a preliminary grinding of the houseflies, followed by further blending in the Potter-Elvehjem homogenizer. This secondary blending led to high yields of mitochondria from whole flies and gave preparations with satisfactory stability. It is possible to wash such preparations twice (about 40 min required) and then to hold the concentrated suspension at 0° for at least 2 h with only a small loss of activity. When isolated thoraces were used in the present study, the secondary blending was found to be unnecessary and the comminution was done entirely with a mortar and pestle.

Oxygen consumption and esterification of inorganic phosphate were measured as described previously[16]. The amount of mitochondrial protein per flask was determined by the method of JACOBS *et al.*[17].

The Tris (Sigma 121), ATP, sodium pyruvate and hexokinase (Type II) were products of Sigma Chemical Company; sodium α-glycerophosphate, 98 % minimum purity, was obtained from Eastern Chemical Corporation; EDTA (practical grade) was obtained from Eastman Organic Chemicals, converted to the disodium salt and recrystallized twice by the method of BLAEDEL AND KNIGHT[18]; crystalline bovine serum albumin was obtained from Armour. Other chemicals used were of reagent grade. Glass redistilled water was used in preparing all solutions.

RESULTS AND DISCUSSION

Isolation and coenzyme requirements of active housefly mitochondria

Preparation from different tissues: In the course of these studies mitochondrial preparations with qualitatively similar properties have been obtained from whole flies, from heads and thoraces, and from the isolated thoraces alone. Preparations from thoraces alone and from whole flies are compared in Table I for their activities with pyruvate and α-glycerophosphate as substrates. The activity with either substrate was essentially the same, regardless of whether whole flies or the isolated thoraces were used as a starting material. These results are consistent with the idea that the isolation procedure gives a preparation consisting predominately of the so-called "giant mitochondria"[19] of the thoracic flight muscle, regardless of the other tissues present in the starting material.

TABLE I

ACTIVITIES OF MITOCHONDRIAL PREPARATIONS FROM WHOLE FLIES AND ISOLATED THORACES

Each flask contained 100 μmoles Tris, 10 μmoles ATP, 150 Kunitz-McDonald units of hexokinase, 23 μmoles $MgCl_2$, 100 μmoles glucose, 250 μmoles sucrose, 100 μmoles potassium phosphate, 3 μmoles EDTA, either 30 μmoles pyruvate, 30 μmoles pyruvate plus 1–2 μmoles fumarate, or 90 μmoles α-glycerophosphate, and either 3 to 9 mg mitochondrial protein from whole flies or 5 to 7 mg from isolated thoraces. The pH was 7.4; total fluid volume, 3.2 ml including 0.2 ml of 20% KOH in the center well. Incubated 30 min at 25°. The values given are averages, with the range in parentheses.

Starting material	*Number of experiments*	*Substrate*	*QO_2(25°)*	*P/O*
Whole flies	7	Pyruvate	100 (80–120)	2.65 (2.26–3.04)
Thoraces	2	Pyruvate	75 (72– 78)	2.80 (2.76–2.85)
Thoraces	3	Pyruvate/fumarate	114 (87–134)	2.98 (2.68–3.16)
Whole flies	6	α-glycerophosphate	78 (60–102)	1.21 (0.89–1.45)
Thoraces	4	α-glycerophosphate	82 (50–110)	1.12 (0.70–1.50)

Isolation media: Sucrose media, varying in concentration from 0.20 *M* to 0.36 *M* and frequently containing other constituents, have been used for isolating insect mitochondria capable of oxidative phosphorylation[1,2,9,20,21]. WATANABE AND WILLIAMS[22], in their early morphological studies, found that a "fuzzy degeneration" took place in sucrose media and recommended a medium of 2.5% serum albumin in 0.16 *M* phosphate buffer as optimal for preserving the mitochondrial structure. Although a complete survey of media for isolating mitochondria was beyond the scope of this investigation, certain comparisons were made in an attempt to improve the quality of our preparations and to reproduce the results of other investigators. The medium described previously[16] was found to be the most satisfactory of those studied. Use of this medium led routinely to preparations which catalyzed rapid oxidation of pyruvate and gave high P/O ratios without the addition of serum albumin to the incubation medium.

Particular comparison was made between our standard isolation medium and a medium containing 0.25 M sucrose and 0.005 M EDTA (see ref. 9). Mitochondria isolated in the latter medium usually gave somewhat lower Q_{O_2} values than did our standard preparations. Five preparations obtained with the sucrose–EDTA medium were tested with α-glycerophosphate as substrate; in two of these, no phosphorylation was obtained with α-glycerophosphate unless serum albumin (2 %) was included in the incubation medium. These results are similar to those reported by SACKTOR, O'NEILL AND COCHRAN[23] who used the sucrose–EDTA medium. However, the P/O ratios which we obtained with pyruvate as substrate were similar to those obtained with our standard preparations. The preparations made in sucrose–EDTA also gave lower acceptor ratios than will be shown below for mitochondria isolated with the usual medium[16] and, in some cases, showed no evidence of respiratory control when α-glycerophosphate was used as the substrate.

The effects of including phosphate and Tris in the isolation medium were tested in early experiments. In these experiments, whole flies were homogenized in an initial medium containing 0.25 M sucrose, 0.003 M EDTA and 0.006 M each of α-ketoglutarate, citrate, succinate and pyruvate. The homogenate was divided immediately into two or more aliquots. Mitochondria were isolated from one of the aliquots and tested as a control preparation. Phosphate, Tris, or a combination of the two were added to other aliquots of the homogenate, following which the mitochondria were isolated and compared to the control preparation. The results of such experiments are shown in Table II. It can be seen that including either 0.05 M Tris or 0.1 M phosphate in the isolation medium gave greatly increased activity, especially with respect to the amount of phosphate esterified. The addition of both Tris and phosphate gave higher activity than was achieved with either alone.

We have not studied the effect of omitting EDTA, Mg^{++}, and substrates from our

TABLE II

EFFECT OF TRIS AND/OR PHOSPHATE IN THE MEDIUM FOR ISOLATING MITOCHONDRIA FROM WHOLE FLIES

The flask contents were as described in Table I except as follows: 175 μmoles Tris, 200 μmoles glucose, 1110 μmoles sucrose, 1.6 mg DPN, 0.78 mg cytochrome *c*, 1.88 mg TPP, 0.4 mg TPN, 0.2 mg CoA and 2.4 μmoles each of α-ketoglutarate, citrate and succinate per 3.0 ml of medium. In Expts. 1, 2 and 3 each flask also contained 22.4 μmoles of pyruvate; in Expts. 4 and 5, 12.4 μmoles of pyruvate. The initial isolation medium contained 0.25 M sucrose, 0.003 M EDTA and 0.006 M each of α-ketoglutarate, citrate, succinate and pyruvate and was adjusted to pH 7.4. Where added to the isolation medium the Tris was at 0.05 M and phosphate was at 0.1 M. Incubated 60 min at 25°. All values are averages from duplicate flasks.

Exp. number	*Added to initial isolation medium*	*O_2 consumed (μg atoms)*	*Phosphate esterified (μmoles)*	*P/O*
1	—	17.4	6.3	0.36
	Phosphate	25.4	38.4	1.51
2	—	18.3	10.6	0.58
	Phosphate	21.0	25.9	1.23
3	—	12.3	3.1	0.25
	Tris	25.1	25.3	1.01
	Tris, phosphate	26.0	41.0	1.58
4	Tris	25.4	16.3	0.64
	Tris, phosphate	21.5	29.4	1.37
5	Tris	10.8	8.4	0.78
	Tris, phosphate	21.6	33.8	1.56

homogenizing medium. However, ZIEGLER, LESTER AND GREEN[24] found that incubation of mitochondrial fractions in a medium containing 0.003 M $MgCl_2$, 0.003 M EDTA, 0.006 M malate and 0.0003 M DPN restored phosphorylation which had been lost during storage or extensive washing. Their report suggests that EDTA, Mg^{++}, and substrates may exert protective effects during the isolation of mitochondria. KLINGENBERG AND BUCHER[25] have found that their mitochondrial preparations showed respiratory control only when the isolation medium contained at least 0.001 M EDTA. If Mg^{++} was present at a higher concentration than EDTA, only uncoupled mitochondria were obtained.

Cofactor requirements: Initial studies made with preparations isolated in a medium without Tris or phosphate led to the routine inclusion of a cofactor solution (DPN, TPP, TPN, CoA, and cytochrome *c*) in the incubation medium, since the mixture greatly stimulated respiration and phosphorylation with only a small decrease in the P/O ratio. In later experiments, using mitochondria isolated in our standard medium[16], the cofactor mixture was no longer found to be stimulatory and was therefore omitted in the experiments to be described here.

The properties of housefly mitochondria

Pyruvate oxidation: Since early studies by WATANABE AND WILLIAMS[4] and by SACKTOR[2] had indicated that the citric acid cycle is operative in thoracic mitochondria from houseflies, only supplementary evidence for the complete oxidation of pyruvate was sought in this investigation. In a number of experiments it was found that succinate, α-ketoglutarate and glutamate were oxidized by the housefly mitochondria at rates which averaged 42 %, 31 % and 55 %, respectively, of the rate obtained with pyruvate. In other experiments the addition of only 3 μmoles of pyruvate to our standard phosphorylation medium, or the addition of 10 to 20 μmoles to a system containing ATP and Mg ($8 \cdot 10^{-3}$ M) but no hexokinase, led to oxygen uptake values corresponding to 75 to 95 % of the amounts required for complete oxidation of the added substrate. The oxidation of pyruvate (0.01 M) in these preparations is abolished completely by an equimolar concentration of malonate. While these results do not give absolute proof that every step of the citric acid cycle is operative, they suggest strongly that pyruvate is oxidized completely in the housefly mitochondria and that the citric acid cycle is the probable route.

Comparison of activities with pyruvate and α-glycerophosphate: In order for oxidative phosphorylation to function effectively as a source of energy for flight, respiration must take place at a rapid rate (high Q_{O_2}) and be efficiently coupled to phosphorylation (high P/O ratio). Therefore, these two ratios were used as criteria of the activity of the mitochondrial preparations from the standpoint of energy production. As shown in Table I the insect mitochondria have a high Q_{O_2}, and the P/O ratio is near the theoretical maximum, in the case of pyruvate oxidation. The Q_{O_2} values in Table I are comparable to those obtained with mammalian mitochondria[26, 27] and the energy production rate ($Q_{O_2} \times$ P/O) with pyruvate is comparable to the best values obtained by HOLTON *et al.*[27] for heart sarcosomes and higher than those found by the same authors for rat-liver mitochondria.

The Q_{O_2} values in Table I are in sharp contrast to those reported by CHANCE AND SACKTOR[10]. The rates given for oxidation of α-glycerophosphate, obtained by the manometric method, are about one-third as high as CHANCE AND SACKTOR obtained using the platinum electrode and a phosphorylation medium. It is possible that our preparations would yield higher Q_{O_2} values for α-glycerophosphate oxidation if a platinum electrode were available for short-period measurements. With pyruvate as substrate, however, the Q_{O_2} values given in Table I and obtained manometrically are 20 to 25 fold as high as the Q_{O_2} of 4 or 5 implied by CHANCE AND SACKTOR[10]. There appears, therefore, to be a significant difference between the mitochondrial preparations used by CHANCE AND SACKTOR and those used in the present study. The greatest quantitative difference is not that our preparations fail to oxidize α-glycerophosphate rapidly but that the preparations used by CHANCE AND SACKTOR, for unknown reasons, apparently oxidized pyruvate very slowly. Since the oxidation of pyruvate by our preparations yielded usable energy at a rate (Q_{O_2} × P/O) nearly three times that for α-glycerophosphate, it would appear to be premature to conclude that α-glycerophosphate is the principal substrate activating the respiratory chain during flight[10]. The possibility does exist that the live insect uses α-glycerophosphate to initiate flight and then utilizes pyruvate for energy production once flight is under way.

Respiratory control and the effect of serum albumin: The degree to which respiration in a mitochondrial preparation is controlled by the availability or the concentration of a phosphate acceptor may be measured by several methods[28–30] and obviously the extent of control indicated may depend upon the exact conditions under which this test is made. In this investigation we used, as the primary measure of respiratory control, the ratio of the rates of respiration obtained manometrically in the presence and in the absence of added phosphate acceptor, this ratio being referred to as an acceptor ratio. A high acceptor ratio (from 4 to 10), particularly when associated with a high P/O ratio, may be regarded as an indication that respiration is "tightly-coupled" with phosphorylation and controlled by the availability of a phosphate acceptor[28, 29].

As shown in Table III the acceptor ratios obtained with pyruvate do indicate that respiration in insect mitochondria is dependent upon the availability of a phosphate acceptor. For these experiments, ATP and the hexokinase system were used to provide a continuously regenerated supply of ADP. Under these conditions, addition of 1 µmole of ATP per flask gave Q_{O_2} and P/O values as high as those obtained with 10 µmoles, and only moderate reductions in these values were noted with as little as 0.3 µmole of ATP per flask. Despite this, addition of ATP gave the large increases in the rate of pyruvate oxidation noted in Table III. Respiratory control was present

TABLE III

ACTIVITY AND RESPIRATORY CONTROL IN HOUSEFLY MITOCHONDRIA

Each flask contained hexokinase, either 30 µmoles of pyruvate plus 1 to 2 µmoles of fumarate or 90 µmoles of α-glycerophosphate, and 5 to 7 mg mitochondrial protein from isolated thoraces. Ten µmoles of ATP were added to obtain respiratory rates and P/O ratios in the presence of a phosphate acceptor; the ATP was omitted in other flasks to obtain respiratory rates in the absence of an acceptor. The flask contents were otherwise as described in Table I. Incubated 20–30 min at 25°. The values given are averages with the range in parentheses.

Number of experiments	*Substrate*	*QO_2 (with ATP)*	*P/O*	*Acceptor ratio**
5	Pyruvate + fumarate	97 (72–134)	2.87 (2.68–3.11)	12.2 (5.9 –24.7)
3	α-glycerophosphate	72 (50– 89)	1.11 (0.70–1.48)	1.73 (1.41– 1.92)

* Ratio of respiratory rates in the presence and in the absence of added ATP.

even when α-glycerophosphate was used as substrate, though the acceptor ratios were not as impressive as those obtained with pyruvate. This difference may indicate that in our preparations electron transport from DPNH to flavoprotein is more tightly coupled to phosphorylation than is true of the other two sites in the respiratory chain at which ATP is formed. Similar differences between the acceptor ratios, obtained with pyridine nucleotide-linked substrates and with substrates such as succinate and α-glycerophosphate, have been reported by others[13, 25, 27, 31].

The acceptor ratios given in Table III for pyruvate oxidation are substantially higher than previously reported for insect mitochondria with pyridine nucleotide-linked substrates[13, 14] but even the highest ratios are only about one-half the figure obtained for intact insects[3]. The acceptor ratios given here for α-glycerophosphate oxidation are somewhat lower than those found by BUCHER *et al.*[13] for locust mitochondria. This group found that the respiratory control of pyruvate oxidation is more stable to aging than is the case for α-glycerophosphate oxidation. They also found that with prolonged aging the respiratory activity with pyruvate and citric acid cycle intermediates is lost, without impairment of the rate for α-glycerophosphate. In BUCHER's preparations the highest control ratios with α-glycerophosphate were obtained with mitochondria with a high content of endogenous substrate[13, 25]. In contrast, the thoracic mitochondria used in these studies had an insignificantly low endogenous respiration (usually zero).

Recently CHANCE AND BALTSCHEFFSKY[30] have suggested that a respiratory-control ratio, defined as the respiratory rate with ADP divided by the rate after added ADP has been phosphorylated, is a more critical test for respiratory control. We do not believe that this criterion is always suitable as a test for respiratory control, because a high ratio would require not only that respiration be controlled by the availability of ADP but in addition that the ATPase activity in the preparation be minimal. If a preparation contained a highly active ATPase (which could be unrelated to the coupling mechanism) this would, in the presence of ATP, provide a continuously regenerated supply of ADP. Thus, the test suggested by CHANCE AND BALTSCHEFFSKY could result in a comparison of respiratory rates with two effectual concentrations of ADP. Their test would appear to be suitable for showing that respiration is controlled by the ADP concentration but inconclusive for proving the absence of such control.

Despite the objection mentioned above, it was considered pertinent to determine whether or not our preparations would show evidence of respiratory control even when measured as CHANCE AND BALTSCHEFFSKY[30] suggested. Since an oxygen electrode was not available for these studies a single addition of 20 μmoles of ADP was used in manometric measurements of respiratory rates before and after phosphorylation of the added ADP. The results of a typical experiment are shown in Fig. 1. Comparison of the respiratory rate in the presence of ADP (12–18 min) with the rate in the presence of ATP (28–50 min) indicates a respiratory-control ratio[30] of approx. 10. The oxygen uptake from the time of adding ADP until the terminal rate was established (28 min) corresponds to a P/O ratio of 2.8, assuming complete phosphorylation of the added ADP. It was observed in such experiments that a Mg^{++} concentration of $10^{-5} M$ permitted demonstration of high respiratory-control ratios. Omission of Mg^{++} led to low respiratory rates in the presence of ADP. With Mg^{++} at $10^{-4} M$ the respiratory rate did not decline during the experiments, presumably because ADP was regenerated continuously by the Mg^{++}-stimulated ATPase, which we have demonstrated in these preparations. Serum albumin was added to the incubation medium in these experiments to reduce possible ATPase stimulation by even small amounts of the endogenous uncoupling agent[32] which might be formed during the incubation. Even a small amount of ATPase activity would lower the apparent respiratory-control ratio by maintaining a low concentration of ADP during the terminal part of such experiments. When serum albumin was omitted in these experiments, respiratory-control ratios of approx. 5 were obtained.

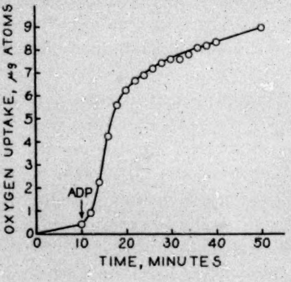

Fig. 1. Effect of ADP on respiration in mitochondria from housefly thoraces. Each flask contained 100 μmoles Tris, 1 μmoles ATP, 0.03 μmole $MgCl_2$, 250 μmoles sucrose, 100 μmoles potassium phosphate, 3 μmoles EDTA, 30 μmoles pyruvate, 2 μmoles fumarate, 45 mg serum albumin, and 7.6 mg mitochondrial protein. The pH was 7.4; total fluid volume, 3.2 ml including 0.2 ml of 20% KOH in the center well. Incubated at 25°. 20 μmoles of ADP were tipped in at the time indicated. Each point is the average of results from two flasks.

Respiratory control has thus been demonstrated in our preparations of washed housefly mitochondria through the use of two different procedures. These results are similar to those obtained by BUCHER *et al.*[13, 25] who used mitochondria from locust flight muscle. It would appear that the mechanism of respiratory control in insect muscle may be the same as it is in other animals which have been studied, contrary to the proposals of CHANCE, SACKTOR AND ESTABROOK[10, 12].

The reasons for the differences in oxidative activity and respiratory control between our preparations and those made by SACKTOR *et al.*[9, 10, 12], from housefly muscle tissue, are not understood completely. SACKTOR's mitochondrial preparations are capable of high P/O ratios but their respiration appears to have been released from dependence on a phosphate acceptor. Recently REMMERT AND LEHNINGER[28] have described a "releasing" factor, derived from mitochondria, which is capable of transforming mitochondrial respiration from the "tightly-coupled" to the "loosely-coupled" state. Many authors have shown the presence, in animal[32–34] and in insect[21] mitochondria, of an uncoupling agent or agents. The actions of both the releasing and uncoupling factor(s) are prevented by including serum albumin in the medium giving, with certain insect preparations, increased oxygen or phosphorus uptake, or both[1, 21]. SACKTOR, O'NEILL AND COCHRAN[23] have reported an absolute requirement for a high level of serum albumin for phosphorylation in housefly mitochondria and suggested that this may reflect the presence of uncoupling factors in their preparations. The presence of uncoupling factors might partially explain the lack of respiratory control which was also observed with SACKTOR's preparations[9, 10]. However, it should be noted that these preparations did not show respiratory control even in the presence of a high concentration of serum albumin.

The effect of serum albumin on oxidative phosphorylatin in our preparations of housefly mitochondria has been tested using both pyruvate and α-glycerophosphate as substrate. As shown in Table IV, the added protein gave slight or insignificant increases in the P/O ratio when pyruvate was used as substrate. With α-glycerophosphate, the added albumin always stimulated oxidation and phosphorylation and led to higher P/O ratios. However, with neither substrate was serum albumin required to obtain substantial phosphorylation and, as shown in Table III, high acceptor ratios are obtained with our preparations without the addition of serum albumin.

TABLE IV

EFFECT OF SERUM ALBUMIN ON OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM HOUSEFLY THORACES

The flask contents were as described in Table I except that serum albumin was added where indicated. All values are averages from duplicate flasks.

Exp. number	*Serum albumin concentration (%)*	*O_2 consumed (μg atoms)*	*Phosphate esterified (μmoles)*	*P/O*
		With pyruvate as substrate		
141	0	21.5	59.4	2.76
	2	16.7	46.5	2.78
150	0	20.0	58.0	2.90
	2	20.4	61.5	3.01
152	0	21.0	62.0	2.95
	2	20.6	66.6	3.23
		With α-glycerophosphate as substrate		
141	0	14.8	17.2	1.16
	2	15.9	20.1	1.26
145	0	11.7	11.9	1.02
	2	14.7	16.4	1.12
147	0	8.7	8.0	0.92
	2	13.0	17.4	1.34

The most striking difference between our method of isolating flight muscle mitochondria and that used by SACKTOR is in the composition of the isolation medium; our medium being more complex, distinctly hypertonic, and well buffered. The mitochondria prepared in the two media differ markedly concerning respiratory control, as already noted. A parallel exists in investigations on *Locusta* mitochondria. REES'[20] earlier preparations of locust flight muscle mitochondria showed quite high P/O ratios but no dependence of respiration on the presence of ADP. These preparations were made in 0.20 *M* sucrose alone. The recent work of BUCHER's group[13, 25] has shown that locust mitochondria do show respiratory control when isolated in a medium containing 0.30 *M* sucrose, 0.002 *M* EDTA and buffered at pH 7.2 with 0.01 *M* triethanolamine. Thus, it would appear that the medium and also the technique employed in isolating insect mitochondria may largely determine the Q_{O_2} and P/O values with different substrates, the requirement for serum albumin, and the degree of respiratory control which are observed with such preparations.

It can be seen that although it appeared only a few years ago, from the evidence then available, that insect mitochondria were "unique" in their ability to oxidize α-glycerophophate at many times the rate with pyruvate or citric acid cycle intermediates, in the mechanism of respiratory control, in their requirement for serum albumin and in their low P/O ratios compared to mammalian mitochondria, this view may no longer be tenable. Recent work has demonstrated that insect mitochondria can metabolize pyruvate and citric acid cycle intermediates at rates comparable with that of α-glycerophosphate oxidation and with high P/O ratios; that they do not always require serum albumin and that they may utilize the same respiratory control mechanism which is found in other species. In fact, flight muscle mitochondria resemble mammalian heart mitochondria with respect to these properties as well as with respect to their ATPase pattern[35] and the composition of their respiratory chains[25, 36].

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THE ENZYMIC INCORPORATION OF THE β-CARBON OF SERINE INTO DIHYDROXYACETONE

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SUMMARY

Dihydroxyacetone is formed from the β-carbon of serine acting, *via* serine aldolase action, as the 1-carbon acceptor for transketolase with either hydroxypyruvate, fructose-6-phosphate or L-erythrulose as donors. The results obtained are considered in relation to the *in vivo* incorporation pathways of ^{14}C into glycogen from [3-^{14}C]hydroxypyruvate and [3-^{14}C]serine.

INTRODUCTION

In 1958, formaldehyde was shown, by DICKENS AND WILLIAMSON[1], to act effectively as an acceptor for transketolase with hydroxypyruvate giving dihydroxyacetone and carbon dioxide. A new route was suggested for carbohydrate synthesis from L-serine via serine aldolase giving formaldehyde (and glycine) which might react with transketolase and hydroxypyruvate formed from L-serine by a specific transaminase[2].

This communication describes conditions under which the β-carbon of serine becomes incorporated into triose in the presence of hydroxypyruvate and partially purified preparations of serine aldolase and transketolase.

MATERIALS AND METHODS

Transketolase was prepared from dried yeast (Distillers Co. Ltd.) by the procedure of DE LA HABA, LEDER AND RACKER[3] omitting the stages of treatment with protamine and alumina. The preparation (10,000 units/ml; 1450 units/mg protein) was free of carboxylase activity tested by measuring carbon dioxide production from hydroxypyruvate (10 µmoles) in the presence of thiamin pyrophosphate (0.05 mg), magnesium chloride (10 µmoles), phosphate buffer (pH 6.2; 53 µmoles) and enzyme preparation (0.2 ml) in a total volume of 1.2 ml.

Serine aldolase was prepared by the procedure of BLAKLEY[4] from an acetone powder of rabbit liver. The enzyme was assayed by determining the amount of serine produced in a system (2.3 ml) containing potassium phosphate buffer (pH 7.5; 40 µmoles), enzyme preparation (0.05 ml), formaldehyde (30 µmoles), glycine (30

Abbreviations: FH_4, tetrahydrofolic acid; BAL, 2,3-dimercaptopropanol; hFH_4, hydroxymethyl FH_4.

μmoles) and FH_4 (2 μmoles) incubated for 30 min at 37° in an atmosphere of N_2–CO_2 (95:5 %). The serine was estimated by heating the incubation systems for 2 min at 100° and measuring, in a Warburg manometer, the amount of CO_2 produced from a 1-ml aliquot *plus* potassium phosphate buffer (pH 5.8; 500 μmoles) treated with sodium metaperiodate (150 μmoles) in a total volume of 2.3 ml. The gas evolution was measured in air and was usually complete in 20 min. The serine aldolase preparations used in the coupling experiments for the synthesis of triose, showed only low serine synthesizing activity in the absence of added tetrahydrofolic acid.

Tetrahydrofolic acid was prepared from pteroylglutamic acid (20 μmoles) by catalytic hydrogenation at 37° in a Warburg manometer; the system (1.55 ml) contained potassium phosphate buffer (pH 6.2; 66 μmoles) and platinum oxide (5 mg) in an atmosphere of hydrogen. The catalyst was saturated with hydrogen before the solution of pteroylglutamic acid was tipped from a side-bulb. The hydrogenation was complete in 30 min, when 1.5 μmoles of BAL, were added. The mixture was quickly filtered and the filtrate stored in an evacuated Thunberg tube at 0°.

The dihydroxyacetone, formed in the incubation systems (3.0 ml) from the coupling experiments, was isolated with carrier dihydroxyacetone (100 μmoles) by the following procedure. At the end of the incubation time, trichloroacetic acid (0.4 ml; 50 %) was added and the supernatant from centrifuging made up to 10 ml with water. After treatment with Amberlite IR 120 (H^+) to remove unchanged serine and Biodeminrolit G to remove other ions, the solution was concentrated under reduced pressure. The dihydroxyacetone was converted to the bis-2,4-dinitrophenylhydrazone of methylglyoxal by distilling the solution (containing approx. 100 μmoles triose) with water (5 ml) and sulphuric acid (1 ml; sp. gr., 1.84) into a saturated solution (15 ml) of 2,4-dinitrophenylhydrazine in 2 *N* HCl. The derivative was collected by centrifugation, plated and counted, if radioactive.

Radioactivity was measured at infinite thickness with correction, where necessary, for self-absorption on 1 cm^2 discs in polythene planchettes; details were identical with those described by DICKENS AND WILLIAMSON[5].

L-[3-^{14}C]serine was supplied by the Radiochemical Centre, Amersham, Bucks, England, and the radioactivity of the solutions used was determined by persulphate oxidation of an aliquot and subsequently counting the barium carbonate prepared from the CO_2 produced[6].

Recrystallised lithium hydroxypyruvate monohydrate was prepared by the method of DICKENS AND WILLIAMSON[7]. L-erythrulose was prepared by the action of *Acetobacter suboxydans* NCIB 7069 on erythritol[8] and isolated as the *o*-nitrophenylhydrazone. Solutions of L-erythrulose were prepared by refluxing the hydrazone with benzaldehyde and a little benzoic acid followed by ether extraction of these substances from the cooled filtrate.

RESULTS

Comparison of formaldehyde with (a) hFH₄ + formaldehyde and (b) hFH₄ as acceptors for transketolase action

The carbon dioxide evolution from an incubation system identical to that described in METHODS for the assay of transketolase (hydroxypyruvate, 10 μmoles and formaldehyde, 10 μmoles) was compared manometrically with the gas evolution from similar systems in which (a) 17.6 µmoles hFH_4 (prepared by mixing equimolar amounts of FH_4 and formaldehyde[9]) were added and (b) 17.6 µmoles hFH_4 replaced the formaldehyde. Carbon dioxide was readily released in the three systems as compared with the control containing hydroxypyruvate but no formaldehyde or hFH_4 (Fig. 1). In the case where hFH_4 was the only added acceptor, the lower rate of reaction was probably due to a slow rate of release of formaldehyde from reversible combination with tetrahydrofolic acid. The equilibrium constant for the reaction $HCHO + FH_4 \rightleftharpoons hFH_4$ has been reported[10] as $K = 1.3 \cdot 10^4\ M^{-1}$.

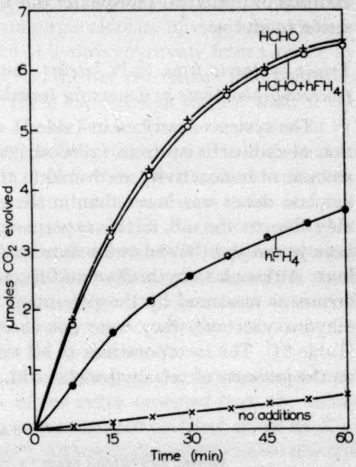

Fig. 1. Release of carbon dioxide from hydroxypyruvate (10 µmoles) in the presence of transketolase (0.2 ml), $MgCl_2$ (10 µmoles), thiaminpyrophosphate (0.05 mg), phosphate buffer (pH 6.2; 53 µmoles); total volume 1.2 ml. ×—×, no additions; +—+, with formaldehyde (10 µmoles); ●—●, with hFH_4 (17.6 µmoles); ○—○, with HCHO (10 µmoles) *plus* h FH_4 (17.6 µmoles). Systems incubated in Warburg manometers at 37°. Gas phase, air.

Triose synthesis from [3-^{14}C]serine (and serine aldolase) with hydroxypyruvate as donor for transketolase

The systems described in Table I were incubated aerobically and the unchanged serine removed by shaking with Amberlite IR 120 (H^+); other ions were removed

TABLE I

TRIOSE SYNTHESIS FROM [3-^{14}C]SERINE AND HYDROXYPYRUVATE

The reaction mixture (3.0 ml) contained hydroxypyruvate (15 µmoles), dihydroxyacetone (100 µmoles), thiamin pyrophosphate (0.02 mg), magnesium chloride (10 µmoles), glycylglycine buffer (pH 7.6; 250 µmoles), L-[3-^{14}C]serine (30 µmoles containing 1.48 µC ^{14}C), serine aldolase (0.2 ml) and transketolase (0.2 ml); incubation: 2 h at 37° in air. The total radioactivity in the dihydroxyacetone isolated from the systems at the end of the experiment was determined.

Additions (µmoles)	*Radioactivity in triose (µC)*	*Radioactivity in triose as % of amount in L-[3-^{14}C]serine*	*Ratio of amount incorporated into triose [0.06 % ≡ 1.0]*
None	0.00093	0.06	1
FH_4 (5 µmoles)	0.103	7.0	114
FH_4 (5 µmoles) + pyridoxal phosphate (0.04 mg)	0.110	7.4	121

subsequently by mixing the systems with Biodeminrolit G. The solutions of dihydroxyacetone were concentrated *in vacuo* and the 2,4-dinitrophenylhydrazones of methylglyoxal prepared from the triose samples as described in METHODS.

The incorporation of radioactivity into the triose during incubation (Table I) was almost entirely dependent on the presence of added tetrahydrofolic acid; the addition of pyridoxal phosphate[4] did not greatly influence the transfer of ^{14}C from serine to glycine.

Triose synthesis from [3-^{14}C]serine (with serine aldolase) and either L-erythrulose or fructose-6-phosphate as donors for transketolase

The systems described in Table II were incubated aerobically and the incorporation of radioactivity from [3-^{14}C]serine into dihydroxyacetone was measured. The amount of radioactivity recovered in the triose with hydroxypyruvate as the transketolase donor was lower than in the previous experiment (Table I). This was probably due to the use, in this experiment, of a serine aldolase preparation which had been frozen and thawed twice since its preparation from an acetone powder of rabbit liver. Although L-erythrulose and fructose-6-phosphate were active as transketolase donors as measured by the percentage incorporation of ^{14}C from [3-^{14}C]serine into dihydroxyacetone, they were not as effective as hydroxypyruvate in this respect (Table II). The incorporations in all three cases were almost completely dependent on the presence of tetrahydrofolic acid.

TABLE II

TRIOSE SYNTHESIS FROM [3-^{14}C]SERINE AND HYDROXYPYRUVATE, FRUCTOSE-6-PHOSPHATE OR ERYTHRULOSE

The reaction mixture (3.0 ml) contained dihydroxyacetone (100 μmoles), thiamin pyrophosphate (0.02 mg), magnesium chloride (10 μmoles), glycylglycine buffer (pH 7.6; 250 μmoles), L-[3-^{14}C]-serine (30 μmoles containing 1.48 μC ^{14}C), serine aldolase (0.2 ml) and transketolase (0.2 ml); incubation: 2 h at 37°. The total radioactivity in the triose was determined at the end of the incubation period.

Additions (µmoles)	*Radioactivity in triose (µC)*	*Radioactivity in triose as % of amount in L-[3-^{14}C]serine*	*Ratio of amount incorporated into triose [0.035% ≡ 1.0]*
Hydroxypyruvate, 15	0.00065	0.044	1.3
Hydroxypyruvate, 15 + FH_4, 5	0.0118	0.8	22.8
Erythrulose, 15	0.00077	0.05	1.4
Erythrulose, 15 + FH_4, 5	0.0044	0.3	8.6
Fructose-6-phosphate, 15	0.00052	0.035	1.0
Fructose-6-phosphate, 15 + FH_4, 5	0.0049	0.33	9.4

DISCUSSION

The results obtained show that serine with serine aldolase (and tetrahydrofolic acid) may provide the 1-carbon acceptor for transketolase action. The dependence of the reactions on the presence of tetrahydrofolic acid indicated that the radioactivity present in the dihydroxyacetone was derived from serine via the serine aldolase reaction

$$\text{Serine} + \text{FH}_4 \xrightleftharpoons[\text{Serine aldolase}]{} \text{Glycine} + \text{h FH}_4$$

$$\text{h FH}_4 \rightleftharpoons \text{FH}_4 + \text{HCHO}$$

$$\text{HCHO} + \text{``Active glycolaldehyde''} \xrightarrow[\text{Transketolase}]{} \text{Dihydroxyacetone}$$

These findings support the possibility stated by Dickens and Williamson[1] that "the route from L-serine to hexose might proceed via serine aldolase (giving formaldehyde and glycine) in conjunction with the formation of hydroxypyruvate from L-serine by the specific transaminase of Sallach[2]." However, the low percentage incorporations from serine into dihydroxyacetone found in these experiments suggest that, if the pathway occurs *in vivo* it is probably a minor one for serine. The incorporation of ^{14}C from [3-^{14}C]serine into liver glycogen *via* dihydroxyacetone would be expected to give glucose labeled equally in carbons 1, 3, 4 and 6, from the results described by Batt, Dickens and Williamson[11] showing that there is no appreciable enzymic discrimination between the two primary alcohol groups of dihydroxyacetone by the kinase which phosphorylates it. However, the distributions of ^{14}C in liver glycogen from both [3-^{14}C]hydroxypyruvate[5] and DL-[3-^{14}C]serine[12] are similar; only a small percentage (4–5 %) of the incorporated ^{14}C from either substrate was recovered in carbons 3 or 4 of the glucose. Most of the radioactivity occurred in carbons 1, 2, 5 and 6. Similar results have recently been reported for L-serine[13].

The incorporation values obtained from the coupling of the serine aldolase and transketolase reactions (Tables I and II) are of the order expected from the values reported for the equilibrium constant of the serine aldolase reaction, given as $K = 2.76 \cdot 10^3\ M^{-1}$ by Alexander and Greenberg[14]. Although the transketolase reaction with formaldehyde as acceptor favours dihydroxyacetone synthesis (Fig. 1), the availability of formaldehyde from serine *via* the serine aldolase reaction presumably limits the rate at which the coupled systems interact.

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ON THE BIOLOGICAL CONVERSION OF GALACTOSE TO GLUCOSE

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SUMMARY

Galactose, talose, gulose and idose labeled in carbon 2 with ^{14}C were synthesized. The conversion of [^{14}C]galactose to glucose isolated from liver glycogen was investigated in the fasted rat. It was found that the bulk of the isotope was in carbon 2 of glucose, indicating that an inositol intermediate could not be involved in the interconversion.

INTRODUCTION

The biological mechanism whereby D-galactose is converted to D-glucose has been the subject of numerous reports. Among these was that of TOPPER AND STETTEN[1] who administered [1-^{14}C]galactose to rats and determined the position of the isotope in glucose isolated from liver glycogen. By this procedure they were able to conclude that conversion had occurred without fragmentation of the carbon chain of galactose. Furthermore, the studies permitted exclusion of most forms of inositol as intermediates. However, mucoinositol, if formed from galactose, can be cleaved in four different positions to yield glucose. As pointed out by the above authors, the finding of isotope predominantly in carbon 1 of glucose eliminated three of these possibilities. The fourth possibility, however, could not be excluded if the assumption were made that the inositol intermediate was continually bound, either to an enzyme surface or in some other manner, thereby destroying the unique symmetry of the molecule.

Since publication of the above report it has been clearly established that the conversion of galactose to glucose occurs while the carbohydrates are bound as UDP derivatives[2]. It was not inconceivable, therefore, that the closing and opening of an inositol ring could have occurred while the carbohydrates were bound in this manner. A postulated mechanism is shown in Fig. 1. This mechanism is based on similar ring openings known to occur in the carbohydrate series[3].

In the present paper the results of an investigation made in order to test this hypothesis are reported. [2-^{14}C]galactose was administered to rats and glucose from liver glycogen was degraded to determine the location of the isotope. From Fig. 1

Abbreviations: UDP, uridine diphosphate; DPN, diphosphopyridine nucleotide.

[*] This work is taken in part from a thesis submitted to the University of Illinois Graduate College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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it can be seen that if the postulated mechanism were operative, the bulk of the isotope would be found in carbon 6 of glucose. It can also be seen that use of [1-^{14}C]-galactose would result in the formation of [1-^{14}C]glucose, in accordance with the results of TOPPER AND STETTEN[1]. Actual findings showed the bulk of the isotope to reside in carbon 2 of glucose, conclusively eliminating the postulated pathway as a possibility.

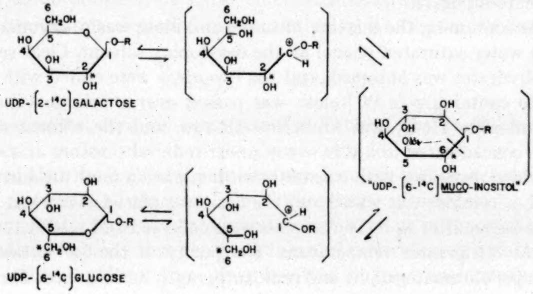

Fig. 1. Hypothetical conversion of UDP-[2-^{14}C]galactose to UDP-[2-^{14}C]glucose. The symbol R represents the uridine diphosphate moiety.

METHODS AND MATERIALS

Preparation of [2-^{14}C]*galactose, talose, gulose and idose*

Strontium xylonate was prepared from commercially obtained D-xylose by the electrolytic oxidation method of ISBELL AND FRUSH[4], and was decarboxylated to D-threose by HOCKETT's modification of the RUFF degradation[5]. Hexoses were prepared from this tetrose by two successive cyanohydrin syntheses[6] using the modifications of ISBELL *et al.*[7]. In the first cycle, Na^{14}CN, obtained from Tracerlab, Inc., Boston, Mass., was employed, and resulted in the formation of [1-^{14}C]xylose and [1-^{14}C]lyxose. The mixture was not separated, but was subjected to a second cyanohydrin synthesis using unlabeled NaCN. A mixture of [2-^{14}C]galactose, talose, gulose and idose was obtained.

The mixture of hexoses was separated by large scale paper chromatography and eluted from the paper. Radioautograms served to locate the sugars on the paper. An initial chromatogram was prepared using *n*-butanol–95 % ethanol–water (7:4:1, v/v/v) as the solvent and developed for 48–72 h. Clear separation of galactose and gulose was achieved, but the talose and idose moved too nearly alike to allow their separation from each other. All three areas were eluted from the paper with water.

The eluate containing [2-^{14}C]galactose was passed over a 1-ml column consisting of a mixture of equal parts of Amberlite IR-45 and Amberlite IR-120 to remove ionic materials eluted from the paper. The effluent and column washings were concentrated to a syrup under reduced pressure at 40°. An equal volume of methanol was added and the solution was refrigerated. Crystallization of galactose occurred, and the material was recrystallized from alcohol. Paper chromatography in three different solvent systems (*n*-butanol–95 % ethanol–water (7:4:1); *tert.*-amyl alcohol–water (11:2); water saturated phenol) and radioautography revealed only one substance present, corresponding exactly in mobility with known galactose. The crystalline material had an equilibrium rotation of $[\alpha]_D^{25} + 79.6°$ ($c = 1$, water).

The eluate containing [2-^{14}C]gulose was treated in the same manner, but crystallization from the syrup could not be achieved. Examination by paper chromatography and radioautography as described above showed only one reducing substance to be present, which was also the only radioactive substance present, attesting to the purity of the carbohydrate.

The eluate containing the mixture of talose and idose was re-chromatogrammed for 24 h using water saturated phenol as the developing solvent. Clear separation of the two carbohydrates was obtained, and the two areas were eluted with water.

The eluate containing [2-^{14}C]talose was passed over a column consisting of a mixture of Amberlite IR-45 and Amberlite IR-120, and the effluent and column washings were concentrated to a thin syrup under reduced pressure at 40°. Absolute ethanol was added dropwise, with constant swirling, until a faint turbidity persisted. A seed crystal of D-talose was added and the flask was placed in a water bath at 9°. Crystallization began after 24 h. The crystals were collected and readily recrystallized from alcohol at refrigerator temperature. The purity of the [2-^{14}C]talose was established by paper chromatography and radioautography as described. The crystalline material had an equilibrium rotation of $[\alpha]_D^{25} + 20.5°$ ($c = 1$, water).

The eluate containing [2-^{14}C]idose was treated in the same manner, but crystallization could not be achieved. The syrup was stored at —15°. Idose was found to be quite unstable, and in other preparations of non-isotopic idose several changes were noted. Syrupy D-idose stored at 4° for some time spontaneously changed to D-sorbose which crystallized out of solution. This transformation is apparently analogous to the conversion of glucose to fructose. Paper chromatography of stored idose preparations frequently revealed the presence of a substance presumed to be a pentose. This assumption was based on the fact that in addition to the idose, a spot having a pink color when sprayed with aniline oxalate was observed, a reaction which has been reported to be typical for pentoses[8]. Idose can be fairly well separated from the pentose by paper chromatography using *tert.*-amyl alcohol–water (11:2), which can be allowed to develop for five days or more. Idose has also been reported to form idosan readily[9].

The rare aldohexoses thus prepared have been used in a variety of metabolic studies, the results of which will be the subject of future reports.

Administration of galactose to rats

Adult male rats of the Sprague-Dawley strain were used as the experimental animals. Prior to the experiment the animals were fasted for 24 h, 4 ml of a 50% aqueous solution of glucose was given by stomach tube, and the desired amount of radioactive galactose was injected intraperitoneally. The animal was immediately placed in a metabolism apparatus which enabled collection of expired CO_2 and urine. After 4 h the animal was sacrificed by a blow on the head and the liver quickly removed.

Isolation of glucose from liver glycogen

Glycogen was isolated from the liver by the trichloroacetic acid extraction method of KARP AND STETTEN[10]. The glycogen so obtained was dissolved in water, dialyzed against water for 24 h at 4°, and precipitated by addition of an equal volume of absolute ethanol. A dry, white material was obtained by successive washings with alcohol and ether in the usual manner. Dialysis was found to facilitate crystallization of glucose in later steps.

Crystalline glucose was obtained by the hydrolytic and isolation procedures described by BELL AND YOUNG[11]. After recrystallization from ethanol, the purity of the product was established by paper chromatography and physical constants. The glucose so obtained was degraded by the method of KOHN AND DMUCHOWSKI[12].

RESULTS AND DISCUSSION

General experimental data are summarized in Tables I and II. Table III shows the results obtained on degradation of the glucose samples derived from liver glycogen. It can be seen that the bulk of the isotope was found in carbon 2, ranging in amount from about 75 to 92 % of the total. This finding made it unnecessary to degrade the molecule completely, since the data are sufficient to allow interpretation.

The findings indicate that the mechanism of conversion of galactose to glucose postulated earlier could not have been involved in the transformation. As pointed

TABLE I

COMPARISON OF ANIMALS ADMINISTERED [2-^{14}C]GALACTOSE

Expt. III was conducted for 2 h. Expts. I and II for 4 h.

	Expt. I	*Expt. II*	*Expt. III*
Animal weight, g	318	404	320
Liver weight, g	9.9	13.5	9.7
Liver glycogen, mg	250	323	105
Glucose from liver glycogen, mg	100	110	36
Expired CO_2 (as g $BaCO_3$)	8.4	9.2	4.9

TABLE II

INCORPORATION OF ^{14}C INTO METABOLIC PRODUCTS OF NORMAL RATS AFTER INTRAPERITONEAL ADMINISTRATION OF [2-^{14}C]GALACTOSE

Experiment number		*Total radioactivity counts/min*	*Radiochemical yield Per cent*
I	Galactose administered	$1.1 \cdot 10^6$	100
	Liver glycogen	$2.3 \cdot 10^5$	20.9
	Expired CO_2	$5.4 \cdot 10^4$	4.9
	Excreted in urine	$1.1 \cdot 10^4$	1.0
II	Galactose administered	$1.1 \cdot 10^6$	100
	Liver glycogen	$3.2 \cdot 10^5$	29.0
	Expired CO_2	$1.9 \cdot 10^5$	17.3
III	Galactose administered	$1.1 \cdot 10^6$	100
	Liver glycogen	$4.5 \cdot 10^5$	40.9
	Expired CO_2	$4.2 \cdot 10^4$	3.8

TABLE III

DISTRIBUTION OF ^{14}C IN GLUCOSE FROM LIVER GLYCOGEN OF NORMAL RATS AFTER INTRAPERITONEAL ADMINISTRATION OF [2-^{14}C]GALACTOSE

Experiment number	*Carbon source*	*Relative specific activity* Counts/min*	*Relative specific activity Per cent*
I	Glucose	2,233	100
	C-1	1,061	7.9
	C-2	10,079	75·3
	C-3,4,5,6**	2,258	16.8
II	Glucose	2,477	100
	C-1	976	6.6
	C-2	13,700	92.3
	C-3,4,5,6**	186	1·3
III	Glucose	10,575	100
	C-1	6,240	9.8
	C-2	54,900	86.6
	C-3,4,5,6**	2,310	3.6

* Specific activity of $BaCO_3$ counted at infinite thickness.
** Obtained by difference (see text).

out, if inositol were an intermediate, the bulk of the isotope would have been found in carbon 6 of glucose. These data are in accord with the more recent theories of the mechanism of interconversion of these two carbohydrates[13], but do not shed any further light on the role of DPN or the formation of a 4-keto intermediate. The findings are also in accord with those of Siu and Wood[14] and Moser and Karnovsky[15]. Similar inferences regarding the role of an inositol intermediate may be drawn from these reports.

ACKNOWLEDGEMENTS

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KINETICS OF PLACENTAL ALKALINE PHOSPHATASE

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SUMMARY

The alkaline phosphatase of human placenta, prepared and purified as described in previous papers[1,2] hydrolyses β-glycerophosphate and phenyl phosphate at nearly the same rate. The alkaline phosphatases of the kidney and the intestinal mucosa, on the other hand, hydrolyze phenyl phosphate much more rapidly than β-glycerophosphate. The Michaelis-Menton constant, K_m, for placental alkaline phosphatase is also different.

INTRODUCTION

In a previous paper[3] we described the preparation and purification of the alkaline phosphatase of the human placenta. By extraction with butanol, fractionation with acetone and with ammonium sulphate, electrophoresis on paper and elution, a highly potent phosphatase preparation was obtained, whose activity appeared to be comparable with the most highly purified phosphatases from other tissues. Selective staining of electrophoretic filter-paper strips with lissamine green for total protein and *p*-nitrophenol (from *p*-nitrophenyl phosphate) for phosphatase seemed to indicate that about one quarter of the total protein was enzymically active. By repeating the electrophoresis on filter paper, elution with distilled water at +4° and dialysis in small cellophane bags, by the technique previously described[3], we have obtained an enzyme which now runs electrophoretically in a single band (Fig. 1). We have not

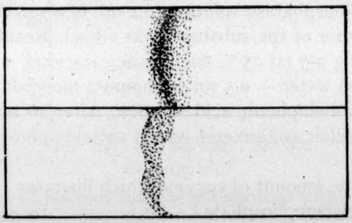

Fig. 1. Paper electrophoretic strips of highly purified placental alkaline phosphatase, prepared by butanol extraction, fractionation, electrophoresis, elution, dialysis and repeated electrophoresis; 1,750 KING-ARMSTRONG units/mg N. Top strip stained with lissamine green, bottom strip with *p*-nitrophenyl phosphate incubated at 37° and exposed to ammonia vapour.

Abbreviation: ATA, Aurine tricarboxylic acid.

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been able to raise its activity above the 1,750 King-Armstrong units previously obtained[3]. This communication describes studies of the chemical composition and kinetic behaviour of this preparation.

The absorption spectra, total and non-protein nitrogen and amino acid content have been measured. Substrate specificity, pH optima, activation and inhibition have been measured and the Michaelis constant determined with several substrates. The kinetic behaviour of the purified placental phosphatase has been compared with those of the kidney and intestinal phosphatases.

MATERIALS AND METHODS

Buffers

Carbonate–bicarbonate buffer: (see ref. 4), veronal buffer: (see ref. 5), glycine buffer: (SÖRENSEN), borate buffer: (PALITSCH). Ethanolamine buffer: ethanolamine was distilled at about 100° *in vacuo*. Buffers of different pH were prepared by the addition of N HCl to a 4 M solution of the ethanolamine.

Substrates

The following esters were used:

Monoesters: α- and β-glycerophosphate, phenyl phosphate, *o*-cresyl phosphate, bromophenyl phosphate, 2,4-dibromophenyl phosphate, ethyl phosphate and glucose-6-phosphate.

Diesters: Diphenyl and diethyl phosphate.

Polyphosphates: Meta- and pyrophosphates.

Enzymes: Purified placental, kidney and intestinal phosphatases were prepared as described in previous papers[2,3].

Estimation of phosphatase activity

Method of KING AND ARMSTRONG[6]; as modified by KING, ABUL FADL AND WALKER[7]. Hydrolysis of phenyl phosphate at pH 10 in Na_2CO_3 buffer.

Method of MORTON[8]: 0.5 ml sodium β-glycerophosphate solution (to give a final concentration of 0.02 M) + 2 ml ethanolamine buffer (pH 9.5 at 38°) (to give a final concentration of 0.04 M) + 0.1 ml magnesium sulphate solution (to give a final concentration of 0.01 M) + 2.3 ml distilled water in a test tube, were heated in a water bath for 3 min. Phosphatase solution (0.1 ml) of a suitable dilution to give not more than 5 % hydrolysis of the substrate was added. Reaction was stopped after exactly 5 min by adding 2.5 ml 25 % trichloroaceticacid. 1 ml of the mixture was taken + 2.3 ml distilled water + 0.5 ml ammonium molybdate (5 % in 5 N H_2SO_4) + 0.2 ml aminonaphthol sulphonic acid solution. After 10 min the blue colour was compared in a photoelectric colorimeter with a suitable phosphate standard treated in the same manner.

MORTON'S *units*: The amount of enzyme which liberates 1 μg of inorganic P/min at 38° from sodium β-glycerophosphate.

Nitrogen: Micro-Kjeldahl digestion followed by Nesslerization[9].

Hydrolysis of placental enzyme

The highly purified enzyme, dialyzed in distilled water overnight at 4°, was hydrolyzed in 4 N HCl in a sealed tube at 120°, for 16 h. The tube was opened, the excess HCl removed by evaporation under reduced pressure, and the residue dissolved in 1 ml of distilled water. A two dimentional chromatogram was run, using No. 1 Whatman filter paper (20 × 20 cm) with phenol and then with collidine. After having been dried the chromatogram was sprayed with 0.1 % ninhydrin solution in butanol–water.

Test for histidine: The two dimensional chromatogram was washed by elution with ethyl acetate, the paper left to dry and sprayed with a mixture of sulphanilic acid and sodium nitrite. After having been left to dry again, it was sprayed with 20 % sodium carbonate solution or was exposed to ammonia vapour. A red colour appeared if histidine was present.

pH measurements: These were made at 37°.

RESULTS

Absorption spectra were measured in the u.v. in a Beckman DU spectophotometer. The enzyme solutions were colourless. Fig. 2 shows the absorption spectra of four preparations, two prepared by autolysis and two by butanol extraction[2]. All curves are similar with a maximum absorption at about 278 mμ, probably due to cyclic amino acids. The low absorption at 260 mμ probably indicates an absence of nucleotide.

ROCHE AND BOUCHILLAUX[10] obtained a similar absorption spectrum for their highly purified intestinal phosphatase; and MORTON[8] for his purified intestinal, milk and kidney phosphatases; also ALVAREZ AND LORA-TAMAYO[11] for a purified kidney phosphatase.

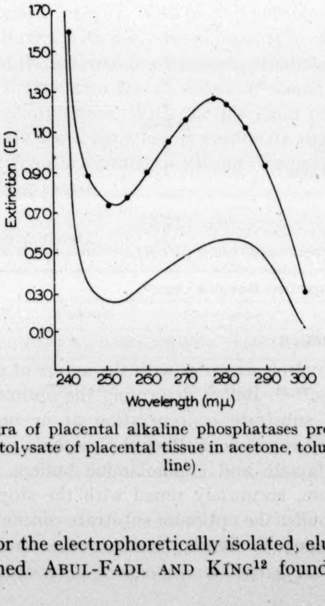

Fig. 2. Absorption spectra of placental alkaline phosphatases prepared from butanol extracts (top line) and from an autolysate of placental tissue in acetone, toluene and ethyl acetate (bottom line).

Chemical composition

Total nitrogen: For the electrophoretically isolated, eluted and dialyzed enzyme, 15.5 % N was obtained. ABUL-FADL AND KING[12] found 10–11 % N for purified faecal phosphatase; MORTON[13] 16.2% N for milk and 15.2% N for intestinal phosphatases.

Carbohydrate: No carbohydrate could be detected with the Molisch reagent. SCHMIDT AND THANNHAUSER[14] reported as much as 21.2% polysaccharide in purified intestinal phosphatase. ABUL-FADL AND KING[15] and ROCHE AND BOUCHILLAUX[16] reported that their intestinal phosphatase preparations were free of carbohydrate. The latter found 12–22% carbohydrate in their initial intestinal phosphatase preparation, but only 1.5–4.4% after purification, due they thought to the elimination of muco-protein. SCHRAMM AND ARMBRUSTER[17] found less than 5% in intestinal phosphatase, MORTON[8] none by the Molisch reaction, but 2% with an anthrone reaction[18].

Amino acids: The amino acids, determined by two dimensional chromatography, are compared with those found by ROCHE AND BOUCHILLAUX[10] for their intestinal phosphatase in Table I. Several amino acids are common to both preparations, but we were unable to show the presence of proline, arginine and histidine.

TABLE I

AMINO ACID CONTENT OF PLACENTAL AND INTESTINAL ALKALINE PHOSPHATASE

Amino acid	*Placental*	*Intestinal**
Valine	+	+
Lysine	+	
Serine	+	+
Glycine	+	
Glutamic	+	+
Threonine	+	+
Alanine	+	+
Leucine	+	+
Aspartic	+	+
Tyrosine	Traces	+
Methionine	?	
Phenylalanine	?	+
Proline	—	+
Arginine	—	+
Histidine	—	Absent or traces

* ROCHE AND BOUCHILLAUX[10].

Optimum substrate concentration

The optimum pH for hydrolysis varies with the nature of the substrate and with the substrate concentration[19, 20]. Before determing the optimum pH, therefore, we determined the optimum substrate concentration at or near the optimum pH determined in a preliminary experiment. Phenyl phosphate and β-glycerophosphate were used with both carbonate and ethanolamine buffers. The duration of the hydrolysis at 37° was 5 min, accurately timed with the stop-watch. With phenyl phosphate and carbonate buffer the optimum substrate concentration was 0.0025 M, and with ethanolamine 0.005 M. Similar results were obtained with β-glycerophosphate.

Optimum pH

The activity curves with the two substrates in carbonate and ethanolamine buffers and also with veronal and glycine were determined. The optima were with phenyl phosphate: carbonate pH 10.3, ethanolamine pH 10.2, veronal pH 9.9, and glycine pH 9.6 (Fig. 3). With β-glycerophosphate they were pH 9.7 in ethanolamine, pH 9.9 in veronal, and pH 9.6 in glycine.

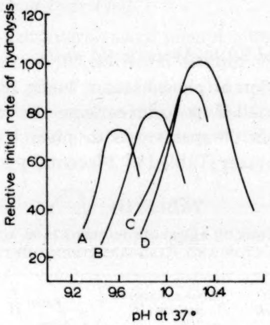

Fig. 3. pH activity curves for the hydrolysis of phenyl phosphate with different buffers. From left to right: glycine, veronal, ethanolamine, carbonate.

The rates of hydrolysis obtained at the optimum pH with the four buffers and both substrates are given in Table II. With phenyl phosphate the highest activity was obtained in carbonate buffer, with β-glycerophosphate in ethanolamine. The addition of magnesium increased the activity in carbonate, ethanolamine and glycine, but not in veronal (Table II). ZITTLE AND DELLA MONICA[21] found that carbonate ions were inhibitory to alkaline phosphatase. With the placental phosphatase and β-glycerophosphate the hydrolysis was a little less in carbonate than in ethanolamine, but in both was much higher than in veronal or glycine. Veronal gave similar results with milk- and intestinal phosphatase.

TABLE II

RATE OF HYDROLYSIS OF PHENYL PHOSPHATE AND β-GLYCEROPHOSPHATE IN DIFFERENT BUFFERS

	mg P liberated/0.1 ml enzyme solution/min							
Substrate	*Carbonate*		*Ethanolamine*		*Veronal*		*Glycine*	
	No Mg^{++}	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*
Phenyl phosphate	(pH 10.3)		(pH 10.2)		(pH 9.9)		(pH 9.6)	
	1.80	2.16	1.36	2.04	0.96	0.98	1.10	1.22
β-glycerophosphate	(pH 9.7)		(pH 9.7)		(pH 9.9)		(pH 9.6)	
	1.50	2.10	1.64	2.24	0.82	0.82	0.86	0.94

Substrate specificity

Like purified kidney and intestinal alkaline phosphatase, that of the placenta hydrolyzed all the monoesters of phosphoric acid tested, *i.e.* α- and β-glycerophosphate, glucose-6-phosphate, ethyl phosphate, phenyl, bromophenyl, dibromophenyl and *o*-cresyl phosphates, but failed to attack diethyl and diphenyl phosphates and meta- and pyro-phosphates.

SCHMIDT AND THANNHAUSER[14] reported that their purified intestinal enzyme hydrolyzed both di- and polyphosphates as well as monoesters, but this is contrary to our experience. The highly purified placental phosphatase failed to show any activity against them at pH values from 7.5–10, using large amounts of enzyme and incubation periods of up to 2 h.

Comparison of the MORTON *and* KING-ARMSTRONG *units*

The ratio differs with different phosphatases, being highest with the placental (Table III). The big differences led us to investigate the rate of hydrolysis of both α- and β-glycerophosphates in comparison with phenyl phosphate by placental, kidney and intestinal phosphatases (Table IV). Placental phosphatase hydrolyzed the

TABLE III

DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY BY THE MORTON AND KING-ARMSTRONG METHODS

Source of phosphatase solution	MORTON *units/ml (I)*	KING-ARMSTRONG *units/ml (II)*	*Ratios* $\frac{I}{II}$	*Average ratios*
Placental:				
Crude	34.7	0.6	57.8	
Purified	98	1.8	54.4	57.6
Purified	14.6	0.24	60.8	
Kidney:				
Crude	9.8	0.36	27.2	26.4
Purified	30.6	1.2	25.5	
Dog intestinal:				
Purified	12	0.7	17.1	17.4
Purified	6	0.34	17.6	
Calf intestinal:				
Purified	33	1.4	23.6	23.8
Purified	60	2.5	24.0	

* β-glycerophosphate as substrate, ethanolamine–HCl buffer pH 9.5; μg P/min at 38°.
** Sodium phenyl phosphate as substrate, Na_2CO_3 : $NaHCO_3$ buffer pH 10; mg phenol/15 min at 37°.

TABLE IV

RATE OF HYDROLYSIS OF α- AND β-GLYCEROPHOSPHATE AND PHENYL PHOSPHATE BY PLACENTAL, KIDNEY AND INTESTINAL ALKALINE PHOSPHATASES

Carbonate and ethanolamine buffers were used.

Enzyme	*mg P liberated/1 ml enzyme solution/15 min*					
	α-Glycerophosphate		*β-Glycerophosphate*		*Phenyl phosphate*	
	Carbonate buffer	*Ethanolamine buffer*	*Carbonate buffer*	*Ethanolamine buffer*	*Carbonate buffer*	*Ethanolamine buffer*
Placental	18	22	21	22	25	22
Kidney	2	4.5	3	7.5	14	9
Intestinal	3.5	10	6.5	20	47	39

three substrates at almost the same rate. Kidney and intestinal phosphatase hydrolyzed phenyl- much more rapidly than β-glycerophosphate, and it more rapidly than α-glycerophosphate, as first shown by KAY[22, 23]. The two buffers carbonate and ethanolamine again gave very similar results with placental phosphatase, but quite different results with the kidney and intestinal enzymes. Fresh aqueous extracts of the three tissues confirmed these differences.

Effect of temperature on rate of hydrolysis

Fig. 4 shows the rates of hydrolysis of phenyl phosphate in carbonate buffer by placental phosphatase from 25–70°, with and without added magnesium. The increase is linear up to 55° without added magnesium, and up to 60° in its presence; and is much steeper in the presence of magnesium, which seems to have a protective effect at the higher temperatures. Similar results were obtained with β-glycerophosphate.

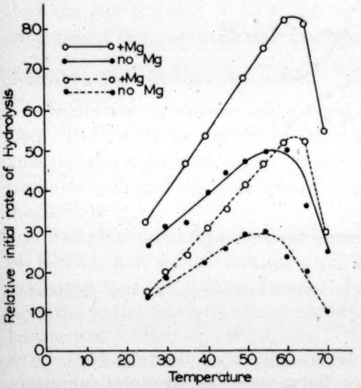

Fig. 4. Effects of temperature on the rate of hydrolysis of phenyl phosphate and β-glycerophosphate by placental alkaline phosphatase. Continuous lines, phenyl phosphate; broken lines, β-glycerophosphate.

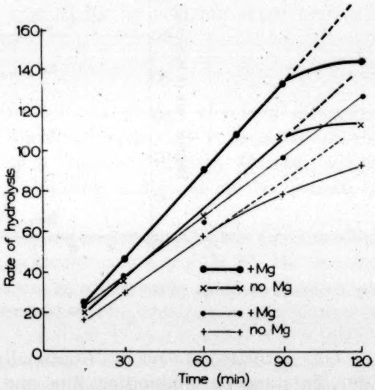

Fig. 5. Effects of time on rate of hydrolysis by placental alkaline phosphatase of phenyl phosphate in carbonate buffer (thick lines) and in ethanolamine buffer (thin lines).

Effect of time on rate of hydrolysis

The hydrolyses were at 37°. With phenyl phosphate in carbonate buffer the rate was linear up to 90 min with and without magnesium. In ethanolamine buffer the rate was linear only to 60 min in the absence of magnesium, while in its presence it remained linear to 120 min (Fig. 5). β-glycerophosphate in carbonate buffer was hydrolyzed in a linear manner for 60 min in the absence of magnesium, and for 90 min in its presence. In ethanolamine buffer the rate was linear for 90 min with and without added magnesium.

Activation and inhibition by cations

Fig. 6 shows the effects of several cations on the hydrolysis of phenyl phosphate in carbonate buffer of pH 10 by the highly purified placental alkaline phosphatase.

Magnesium showed the highest activation at 0.02 *M* concentration. The effect of Mg^{++} is less pronounced with placental phosphatase than with those from several other tissues, and provides further evidence for its difference from them. The smallness of magnesium activation with placental phosphatase was confirmed with other preparations, and was found whether the enzyme was purified or crude, dialysed or undialysed. Although several factors are known to influence the degree of activation of alkaline phosphatases by magnesium (*e.g.* concentration of Mg^{++}, nature of substrate and its concentration, degree of enzyme purity etc., (see ref. 23)) it nevertheless seems that some phosphatases are more activated by Mg^{++} than others.

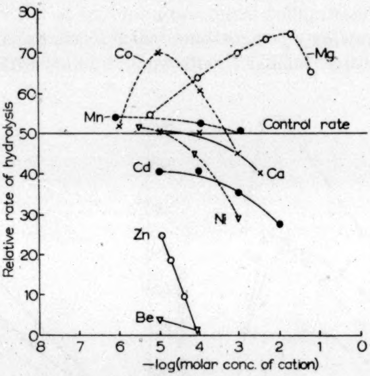

Fig. 6. Effects of cations on the hydrolysis of phenyl phosphate by placental alkaline phosphatase in carbonate buffer.

Co^{++} activated at 0.00001 *M* optimally; Mn was slightly activating; Ni^{++}, Ca^{++}, Cd^{++}, Zn^{++} and Be^{++} inhibiting. Zinc and beryllium were the strongest inhibitors. The effect of adding Mg^{++} to the enzyme solution in the presence of these cations is shown in Table V. With Co^{++} a slight activation is enhanced, and the powerful inhibition caused by Zn^{++} and Be^{++} disappeared. Where the activation was slight, *i.e.* with Ni^{++}, Ca^{++} and Mn^{++}, Mg^{++} had little or no effect.

TABLE V

ACTIVATION BY Mg^{++} IN THE PRESENCE OF OTHER CATIONS

Inhibitor	*Units/ml enzyme solution*	
	No Mg^{++}	*+ Mg^{++}*
Dialyzed enzyme	40	57
0.0001 *M* Co^{++}	52	57
0.0001 *M* Mn^{++}	47	47
0.00001 *M* Ni^{++}	46	48
0.0001 *M* Ca^{++}	41	46
0.0005 *M* Cd^{++}	32	32
0.00002 *M* Zn^{++}	8	57
0.00001 *M* Be^{++}	4	54

The experiment was repeated with ethanolamine buffer, with some differences: Mg^{++} was activating, Co^{++}, Mn^{++}, Ni^{++} and Ca^{++} were slightly activating, and Cd^{++}, Zn^{++} and Be^{++} were inhibiting. With Zn and Be the addition of Mg restored activity, although not fully. With the others nearly complete activity was restored.

The inhibition of alkaline phosphatase by Be^{++} has been extensively investigated by KLEMPERER, MILLER AND HILL[25], who claimed that the inhibition is independent of the nature of the substrate. DUBOIS, COCHRAN AND MAZUR[26] suggested that Be^{++} may interfere with the biological function of calcium and magnesium, for it occurs in the same group in the periodic table. ALDRIDGE[27] found that Mg^{++} can reverse the inhibition by Be^{++}. This agrees with our findings and shows that Be^{++} inhibition is largely due to competition with Mg^{++}. LINDENBAUM, WHITE AND SCHUBERT[28] found that ATA reversed the inhibition induced by Be^{++}. VEERKAMP AND SMITS[29] claimed that the inhibition *in vitro* bears no relation to the action of Be^{++} *in vivo* and that Be^{++} acts *in vitro* by precipitating the enzyme. SCHUBERT AND LINDENBAUM[30] suggested that the effectiveness of ATA can be accounted for by bringing about reversible chelation of Be^{++} with alkaline phosphatase.

Activation and inhibition by anions

Inhibition by anionic and related substances was studied at several concentrations. The results are set out in Table VI. Borate, fluoride, D.F.P., and iodo-acetate and thioglycolate were either uninhibiting or slightly inhibiting. Cyanide, iodine, formaldehyde and arsenate were strongly inhibiting, and could not be reversed by magnesium.

SIZER[31] showed that the inhibition of alkaline phosphatase by low concentrations of $KMnO_4$ and iodine was reversed by reducing agents (*e.g.* H_2S). He therefore suggested that the (OH) groupings of the cyclic amino acids, such as tyrosine, are essential to the catalytic action of the phosphatase. The inhibition of phosphatase by borate was studied by ZITTLE AND DELLA MONICA[21]. They concluded that it inhibits in a way similar to that of phosphate. JACOBSEN[32] showed phosphate inhibits both competitively and non-competitively, and from the general similarity in behaviour of arsenate to phosphate, the inhibition by arsenate is to be expected.

TABLE VI

EFFECT OF SOME ANIONS AND OTHER SUBSTANCES ON THE ACTIVITY OF PLACENTAL ALKALINE PHOSPHATASE

Inhibitor	*Units/ml enzyme solution*							
	None		*0.01 M*		*0.001 M*		*0.00001 M*	
	No Mg^{++}	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*
Dialyzed enzyme	34	49						
+ Borate			25	33	33	49		
Cyanide			6	6	25	25	37	37
Fluoride			35	35	35	35		
DFP (diisopropylfluorophosphate)							34	49
Iodine					6.5	9.5		
Iodoacetate					35	45		
Thioglycolic acid					27	27		
Formaldehyde			3.5	3.5				
Phosphate					18	33		
Arsenate					2	2		

Macroionic inhibition

During our study of placental phosphatase we tried to concentrate an enzyme solution by placing it in a cellophane bag and then leaving it in a 15 % solution of polyvinyl pyrolidone (molecular weight 11,000). After a few hours the enzyme solution in the bag had undergone considerable concentration by volume, but upon testing proved to be without activity.

KATCHALSKI, BERGER AND NEUMANN[33] described the disappearance of pepsin activity when the enzyme was treated with polymers, and HAHN[34] the inhibition of hyaluronidase. DICZFALUSY *et al.*[35] described the inhibition of acid and alkaline phosphatase, among other enzymes, by phosphate polymers of various aromatic hydroxy and amino compounds.

Activation and inhibition by amino acids

All amino acids were used in a concentration of 0.001 *M*. Alanine alone produced slight activation, the rest either had no action or were slightly inhibitory (Table VII).

TABLE VII

EFFECT OF SOME AMINO ACIDS AND OF ASPARAGINE AND GLUTAMINE (ALL IN 0.001 *M* CONCENTRATION) ON THE ACTIVITY OF PLACENTAL ALKALINE PHOSPHATASE

K-A. Units/ml enzyme solution			
Amino acid	*+ Mg⁺⁺*	*Amino acid*	*+ Mg⁺⁺*
None	53	Hydroxy proline	50
Alanine	59	Isoleucine	48
Arginine	45	Ketoglutaric	51
Asparagine	49	Lysine	47
Aspartic	50	Proline	49
Glutamic	52	Serine	54
Glutamine	44	Threonine	53
Histidine	45	Tyrosine	54

THOAI, ROCHE AND ROGERS[36] found that intestinal alkaline phosphatase lost most of its activity on prolonged dialysis, but pre-incubation with magnesium and addition of alanine restored its activity to a level higher than before dialysis. MORTON[13] found no activation of intestinal phosphatase by alanine in veronal buffer, but some in carbonate. The slight inhibition by histidine is in contrast to the activation of kidney phosphatase observed by ABUL-FADL AND KING[15] and AKAMATSU AND KOBAYASHI[37].

Michaelis constants

To determine K_m we used the following equation derived by HOFSTEE[38].

$$V_m = v + \frac{v}{S} K_m$$

Plotting v against v/S one gets a straight line cutting both axes. V_m is given by the intercept with the ordinate.

K_m was determined using phenyl phosphate and β-glycerophosphate as substrates and carbonate buffer (pH 10). Both substrates were used at the following concentrations: 0.005, 0.0025, 0.001, 0.0005 M. The time of incubation was exactly 5 min. Fig. 7 shows the curves obtained using placental alkaline phosphatase. The following K_m values were obtained graphically from figures so drawn. The K_m values for purified kidney and intestinal alkaline phosphatases were similarly determined.

Substrate	*pH*	*Km values*		
		Placental	*Kidney*	*Intestinal*
Phenyl phosphate	10	0.0012	0.0057	0.0021
β-glycerophosphate	10	0.0018	0.025	0.012

These results show quite clearly that while the K_m or substrate affinity of kidney and intestinal phosphatase using phenyl phosphate and β-glycerophosphate are quite different, those obtained with the placental enzyme were nearly the same.

Roche and Sarles[39] found different K_m values for liver, intestinal and bone phosphatase and concluded that such differences may be due to differences in the apo-enzyme. Morton[8] found the K_m of intestinal phosphatase with phenyl phosphate to be 0.00086, while with β-glycerophosphate it was 0.017.

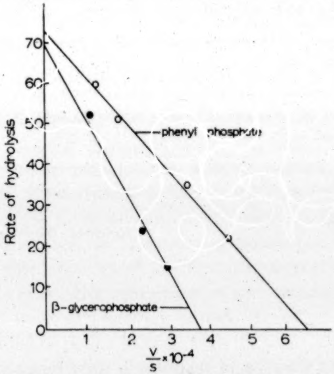

Fig. 7. Evaluation of K_m of placental alkaline phosphatase using phenyl phosphate and β-glycerophosphate as substrates.

On the basis of K_m values Folley and Kay[40] pointed out that the alkaline phosphomonoesterases seemed to have a much greater affinity for phenyl phosphate than for glycerophosphate. Their conclusions are supported by our findings for kidney and intestinal phosphatases, but cannot be extended to include placental phosphatase.

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Short Communications

The endogenous formation of phosphoenolpyruvate by rat-liver mitochondria

Phosphoenolpyruvate has been shown to be formed by kidney and liver particulate preparations in the presence, but not in the absence, of added Krebs-cycle acids[1-5]. During the course of a study of phosphorylated components in mitochondria, we have found that [^{32}P]PEP was formed on incubating mitochondria with $H_3{}^{32}PO_4$ in the absence of added substrate. This report concerns the identity of PEP and its formation under various conditions, and the relation of PEP formation to the endogenous activity of mitochondria.

Rat-liver mitochondria were prepared in 0.25 *M* sucrose as described previously[6]. Incubation was carried out aerobically at 30° in a final volume of 1 ml, with final concentrations as follows: mitochondrial N, 1.5 mg/ml; sucrose, 0.125 *M*; Tris (pH 7.4), 0.05 *M*; $MgCl_2$, 0.005 *M*; $H_3{}^{32}PO_4$, 10^{-4} *M*, 100 μC/ml. Incubation time and other substances or changes are noted in the tables. After the period of incubation the suspension was chilled, mitochondria were sedimented at 11,000 × *g* for 10 min at 3°, and the supernatant was fractionated by anion-exchange chromatography. An 0.8 × 10 cm Dowex-1-X8 chloride column was used; the elution was performed by two gradient elutions[7]. In the first, the upper or reservoir flask contained 500 ml 0.02 *M* HCl and the lower or mixing flask, water. During this elution an unidentified phosphate ester stable to treatment with 1 *M* HCl for 10 min at 100°, phosphate, and a second unidentified ester of similar stability to the first were successively removed. In the second elution the reservoir contained 500 ml 0.02 *M* HCl and 0.3 *M* NaCl; the mixer contained 500 ml of the final concentration from the first elution, approx. 0.013 *M* HCl. Here, the remainder of the second stable ester mixed with ADP, PEP, and ATP were successively eluted. No other labeled substances were found. Fractions included in peaks of radioactivity were pooled and brought to pH 7. The substances were assayed with myokinase or pyruvic kinase in combination with hexokinase and glucose; the [^{32}P]G-6-P which formed was determined[8] as phosphate ester stable to heating for 30 min at 100° in 1 *M* HCl.

The PEP fraction exhibited lability of the phosphate ester to alkaline iodine and neutral $HgCl_2$, and had a half-life of approx. 10 min in 1 *M* HCl at 100°. The product when recrystallized three times as the silver-barium salt with synthetic PEP[9] as carrier, had a specific activity of 2780, 2700, and 2800 counts/min/mg after the respective recrystallizations. [^{32}P]PEP was synthesized according to OHLMEYER[9] using $^{32}POCl_3$ prepared by a modification of the procedure of KALINSKY AND WEINSTEIN[10]. Synthetic [^{32}P]PEP and the mitochondrial product were eluted from the Dowex-1 column in the same fractions, and had identical R_F's on paper chromatography with

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Abbreviations: PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; ADP, adenosine diphosphate; ATP, adenosine triphosphate; G-6-P, glucose 6-phosphate; GSH, glutathione; EDTA, ethylenediaminetetraacetate; ITP, GTP, inosine and guanosine triphosphate.

two different solvent systems. It may be noted here that in the phosphate-ester estimation[8], which depends on the extraction of phosphomolybdate with organic solvent, PEP is extracted into the organic phase to the extent of approx. 40 %.

The incorporation of ^{32}P into ATP and PEP under various experimental conditions is depicted in Table I. Of the uncouplers of oxidative phosphorylation, pentachlorophenol[11] and 2,4-dinitrophenol enhanced the ^{32}P incorporation into PEP and depressed that of ATP, while $CaCl_2$ only depressed ATP incorporation. Aging the mitochondria and inclusion of KCN depressed incorporation into both ATP and PEP, while GSH, EDTA, KF, or omission of $MgCl_2$ had little effect. The influence of the duration of incubation is shown in Table II. In the absence of 2,4-dinitrophenol the labeling of ATP reached a peak at 15 min while that of PEP followed at 35 min, indicating that the ^{32}P of PEP was not a precursor of [^{32}P]ATP. In the presence of 2,4-dinitrophenol the labeling of ATP remained negligible and that of PEP reached a peak at 15 min.

TABLE I

INCORPORATION OF ^{32}P INTO ATP AND PEP

Abbreviations: DNP, 2,4-dinitrophenol; PCP, pentachlorophenol.

Expt.	*Experimental condition*	*Incubation time (min)*	*mμmoles ^{32}P incorporated*	
			ATP	*PEP*
1.	Control	30	0.62	0.46
	$2.5 \cdot 10^{-4}$ *M* DNP	30	0.08	1.41
	0.01 *M* KCN	30	0.04	0.21
	0.001 *M* GSH	30	0.59	0.47
	0.001 *M* EDTA	30	0.82	0.39
2.	Control	10	1.09	0.39
	Omit $MgCl_2$	10	1.17	0.46
	$5 \cdot 10^{-5}$ *M* PCP	10	0.18	1.99
	0.02 *M* KF	10	1.94	0.49
	0.002 *M* $CaCl_2$	10	0.23	0.41
3.	Control	30	0.65	0.42
	Mitochondria aged 24 h at 3°	30	0.22	0.07

TABLE II

EFFECT OF INCUBATION TIME ON INCORPORATION OF ^{32}P INTO ATP AND PEP

Incubation time (min)	*mumoles P incorporated*			
	Control		*$2.5 \cdot 10^{-4}$ M 2,4-dinitrophenol*	
	ATP	*PEP*	*ATP*	*PEP*
0	0.30	0.10	0.06	0.83
5	—	—	0.08	1.86
15	1.13	0.31	0.08	2.31
35	0.43	0.33	0.08	1.55
65	0.12	0.19	0.08	1.06

One mechanism for PEP formation involves the action of oxaloacetic carboxylase on oxaloacetate and ITP or GTP[5,12]. However, we have found no peaks corresponding to [^{32}P]ITP or [^{32}P]GTP, which would be expected to occur if these were precursors of [^{32}P]PEP. The stimulation of endogenous formation of PEP by uncouplers is reminiscent of the observation of MUDGE *et al*[3] that in liver mitochondria 2,4-dinitrophenol stimulated PEP formation in the presence of α-ketoglutarate or its immediate precursors but not in the presence of fumarate, malate, or oxaloacetate. The stimulation of PEP formation appears to be more related to the specific action of 2,4-dinitrophenol and pentachlorophenol rather than to the result of uncoupling of endogenous oxidative phosphorylation, since Ca^{++}, which is a potent uncoupler in intact mitochondria, did not exhibit the effect characteristic of the substituted phenols.

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A new approach to the separation and purification of peptides

The isolation of pure peptides is an important step in the study of protein structure. Mixtures of peptides are often difficult to fractionate because they display only small differences in physico-chemical properties. This communication describes a new approach to the chromatographic fractionation of peptides which is based on small differences in the firmness of binding by the free-base form of cellulosic anion exchangers.

The general procedure is simple and rapid. Peptides in a minimal volume of distilled water are added to a cellulosic anion-exchange column[1,2]. The chromatogram is developed with distilled water followed by distilled water saturated with 1 atm. CO_2 (ref. 3). Certain peptides are retarded only slightly and are eluted with the distilled water. Those which are bound more firmly are eluted with the CO_2 solution*.

Abbreviation: DEAE-, diethylaminoethyl-.

* It would be expected that peptides containing dicarboxylic amino acids would be bound very strongly. This was the case with L-Glu-L-Phe, which was not eluted with CO_2 solutions. On the other hand, the tetrapeptide L-Glu-L-His-L-Phe-L-Arg was eluted with CO_2 solutions.

In general, amino acids are not retarded appreciably by the exchanger*, and therefore are separated ahead of the peptides.

A commercial sample of "chromatographically pure" L-Leu-L-Tyr subjected to the procedure was separated from approx. 10 mole-% tyrosine with which it was contaminated (Fig. 1A)**. D-Leu-L-Tyr, from another source, showed no evidence of tyrosine. A mixture of these two diastereo-isomers was separated on DEAE-cellulose[1], as shown in Fig. 1B. BLACKBURN AND TETLEY[5] reported this separation on Dowex-50, using citrate buffer as eluent, but the present procedure is simpler, provides better separation, and yields salt-free preparations.

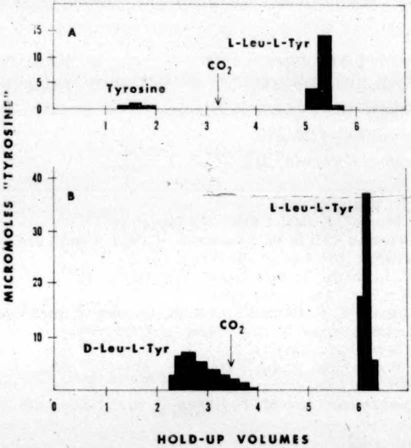

Fig. 1A. Purification of L-Leu-L-Tyr. 20 μmoles of a L-Leu-L-Tyr preparation in 2.0 ml water were added to a DEAE-cellulose column, 37.5 × 0.7 cm. The flow rate was 0.43 ml/min; the fraction volume 3.0 ml; and the hold-up volume, 12 ml.

Fig. 1B. Separation of D-Leu-L-Tyr and L-Leu-L-Tyr. 64.5 μmoles of each diastereoisomer in 5.85 ml water were added to a DEAE-celluiose column 44.5 × 1.1 cm. The flow rate was 1.1 ml/min; the fraction volume, 5.0 ml; and the held-up volume, 40 ml.

The separation of glycine from the di- and tripeptides of glycine on DEAE-cellulose is shown in Fig. 2. Similar separation of glycine and glycylglycine was obtained with Ecteola-cellulose[2].

The binding of a dipolar ion by the free-base form of DEAE-cellulose evidently depends upon the equilibrium between the tertiary amine of the exchanger and the amino group of the dipolar ion.

$$\underset{\text{not bound}}{H_3\overset{+}{N}\text{-R-COO}^- + R'R''_2N} \rightleftarrows \underset{\text{bound}}{H_2N\text{-R-COO}^-R'R''_2NH^+}$$

The relative basicities of the two amines determine the position of this equilibrium

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[*] On a DEAE-cellulose column, asparagine is retarded slightly, cysteine is held to a greater degree, and dicarboxylic amino acids are strongly bound.

[**] Tyrosine and its derivatives were identified by u.v. spectrophotometry, polarimetry, paper chromatography, and the characteristic ninhydrin reaction rates and maximal color yields[4].

which, in turn, determines the degree of binding of the dipolar ion. Studies indicate that the degree of binding of dipolar ions, in general, increases with decreasing basicities of their amino groups. Dipolar ions may be classified into four categories with respect to their relative ease of elution from DEAE-cellulose: (1) those which are not retarded, (2) those which are retarded, but are eluted with distilled water, (3) those which are eluted by a CO_2 solution, and (4) those which are not eluted with CO_2 solutions. The first three of these categories are represented in Fig. 2.

Certain strong anion exchangers, *e.g.*, the methyl quaternary of DEAE-cellulose[6], may be suitable for the chromatography of those dipolar ions which are too readily eluted from DEAE-cellulose for adequate separation, whereas weak anion exchangers, *e.g.*, *p*-aminobenzylcellulose[7], may be used for those which are not eluted from DEAE-cellulose with CO_2 solutions.

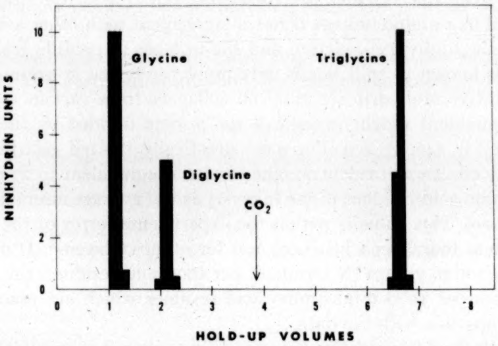

Fig. 2. The separation of glycine, diglycine, and triglycine. 25 μmoles of each compound were mixed in 1.5 ml water and added to a DEAE-cellulose column, 83 × 0.7 cm. The flow rate was 0.15 ml/min; the fraction volume, 3.0 ml; and the hold-up volume, 26 ml. (Glycine and its derivatives were chromatographed singly in order to identify the components in this chromatogram.)

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Guanidination of lysine and hydroxylysine in ichthyocol

LEVY, FISHMAN AND CABRERA[1] reported recently that approx. 35–40 % of the lysine residues in tendon collagen and in ichthyocol are not available for reaction with nitrosyl chloride and they concluded that these lysine ε-amino groups are not free. Evidence for the presence in collagen of ε-amino groups in peptide linkage was provided by the isolation from partial hydrolyzates of a peptide in which the ε-amino group of lysine is bound in this manner[2].

The determination of the precise number of ε-amino groups bound in peptide linkage would be of considerable importance. The possibility that 35–40 % of the lysine residues (8–10 lysine residues per 1000 total amino acid residues) present in collagen are bound in ε-amino linkage is not in agreement with other available data for the extent of the ninhydrin reaction and the number of peptide bonds split by trypsin. Trypsin is known to split bonds only involving lysine or arginine residues. Using the quantitative ninhydrin reaction[3] on collagens from various sources[4], it is found that the equivalent weight (weight of the protein divided by the number of leucine equivalents) is 3400 ± 200. For more drastically treated gelatins the value approaches 3700–4000. An equivalent weight of 3400 is equivalent to 27 residues per thousand total amino acid residues if one takes 93 as the average amino acid residue weight for ichthyocol. This value is within the experimental error of the lysine plus hydroxylysine values found for ichthyocol and for other collagens[5]. If one assumes a maximum of 2 α-amino groups (N terminal) per thousand residues, the estimate of 8–10 lysine residues per 1000 total amino acid residues which are unavailable for reaction is not compatible with the data.

When gelatin derived from ichthyocol or calf-skin collagen is completely digested with trypsin, the average equivalent weight of the peptides formed is 1730 based on the ninhydrin reaction, and 1200 as determined by formal titration[6]. This discrepancy might be expected since the hydrolysis of Lys·Pro, Arg·Pro or the corresponding hydroxyproline-containing linkages would not be accompanied by an increase in ninhydrin color, but would yield the expected increase in the formol titration. In ichthyocol and in calf collagen there are approx. 46 residues of arginine and 24 of lysine. Thus, an equivalent weight of approx. 1300 is obtained by dividing 70 into 93,000. This is in good agreement with the formol-titration data obtained after tryptic digestion, *i.e.*, every bond involving a lysine or arginine residue is split within an error of approx. 2 residues. These data suggest that at most 2–4 lysine residues are unreactive and might have their ε-amino group in some type of linkage, presumably peptide.

The end-group methods discussed yield data which offer inferential evidence concerning the number of bound ε-amino linkages, but cannot provide conclusive proof of the quantitative aspects of this problem. For our study of the lysine residues in collagen, we have chosen the guanidination procedure of HABEEB[7] in which the active guanidinating agent is GDMP. The results of HABEEB[7] have been confirmed in that as much as 94 % of the lysine residues of serum albumin can be converted to homoarginine by this guanidination procedure. When the extent of the reaction and the mildness of the conditions employed are considered, this procedure seems to be

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Abbreviation: GDMP, 1-guanyl-3,5-dimethyl pyrazole nitrate.

the one of choice in determining whether ε-amino groups of lysine might be in covalent linkage.

Ichthyocol (approx. 37 mg) and gelatin (derived from approx. 72 mg ichthyocol by heating at 53° for 1 h) were treated with 0.41 M GDMP (0.5 g in a final volume of 6 ml) at pH 9.5 for 7 days at 5°. Ichthyocol was soluble in the concentration of the reagent used and did not appear to undergo denaturation. In the presence of the reaction mixture, gelatin exhibited a tendency to gel at a low temperature; however, because of continuous stirring and the low concentration of protein, the gel was not firm. After allowing the reaction to proceed for 7 days, the mixtures were dialyzed for 3 days against 0.1 M phosphate buffer (pH 7.4) then against distilled water for 4 days with many changes of the dialyzing fluid. The proteins were hydrolyzed for 18 h at 105° with 6 N HCl in a sealed vial; HCl was removed *in vacuo* and amino acid analyses conducted with the automatic amino acid analyzer of SPACKMAN, MOORE AND STEIN[8]. The pertinent results are summarized in Table I. It should be noted that the only two amino acids which changed significantly in content as a result of the guanidination were lysine and hydroxylysine. 87 % of the lysine of ichthyocol and 94 % of the lysine of gelatin were converted to homoarginine. The hydroxylysine content also decreased and in each case a new double peak was eluted from the short column (15 cm) at a position between ammonia and arginine. The double peak is believed to be hydroxyhomoarginine and its allo form, the products that would be expected from the guanidination of hydroxylysine. It is known that hydroxylysine shows a double peak characteristic of the normal and allo forms if chromatographed on a longer column[9]. While the new double peak observed on the short column has not been definitely established as hydroxyhomoarginine and its allo form by comparison with authentic samples, it appears to account for 75 % of the loss in hydroxylysine content of ichthyocol and 87 % in the case of gelatin. The contents of other amino acids in the treated proteins were within experimental error of those for the control.

TABLE I

CONTENT OF CERTAIN AMINO ACIDS IN GUANIDINATED ICHTHYOCOL AND GUANIDINATED ICHTHYOCOL GELATIN COMPARED WITH ICHTHYOCOL

Amino acid	*Ichthyocol (control)*	*Guanidinated ichthyocol*	*Guanidinated ichthyocol gelatin*
	Residues per 1000 total amino acid residues		
Lysine	22.6	3.1	1.6
Homoarginine	—	21.8	24.4
Hydroxylysine	5.7	1.2	0.4
Hydroxyhomoarginine	—	3.6	2.6

JANUS[10], employing the SAKAGUCHI method for analysis, showed that guanidination of lysine and hydroxylysine residues of bone and hide gelatins with O-methylisourea at pH 10.5 was complete. The equivalent weight per guanidino group formed can be calculated as 1100 in one case and 1250 in another, essentially in agreement with our data indicating few bound ε-amino groups.

From the data in Table I it appears that no more than 2-4 residues of lysine and

1 residue of hydroxylysine are protected from guanidination with GDMP. This is a maximum value and is in contrast to the high value of 8–10 residues calculated from the data of LEVY *et al.*[1] as being unreactive with nitrosyl chloride. It is known that the ε-amino peptide bond of lysine which is present in Bacitracin A is extremely resistant to acid hydrolysis[11,12], therefore if the unreactive ε-amino groups of lysine were bound in peptide bonds, one should have expected a greater quantity of ε-amino peptides of lysine in acid hydrolyzates of gelatin than was found by MECHANIC AND LEVY[2]. The peptide isolated by them accounted for less than 1 % of the lysine content of the protein. Similarly, the detection of a large quantity of free lysine after nitrosyl chloride treatment followed by acid hydrolysis[1] when considered with the reported stability of ε-amino peptide bonds is apparently not compatible with the presence of a large number of ε-amino peptide bonds in gelatin and collagen.

Since nitrosyl chloride gave quantitative destruction of the free amino groups of lysine in ribonuclease[1], it is surprising that so many of these residues in ichthyocol were unaffected by the reagent. This point should be investigated further to determine how the collagen structure renders the ε-amino groups of certain lysine residues inaccessible to the action of nitrosyl chloride under the experimental conditions of LEVY *et al.*[1].

It should be emphasized that while the number of bonds involving the ε-amino groups of lysine appears to be small according to our observations, these could still serve an unique role in cross-linking in collagen. These linkages should be sought by attempting to isolate peptides in the manner described by MECHANIC AND LEVY[2].

The observation that hydroxylysine can be guanidinated indicates the presence of this amino acid residue in the protein in a form in which the ε-amino group is readily accessible. Possible formation of hydroxylysine as a hydrolysis product of a cyclic structure, such as an oxazoline, appears unlikely.

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Preparation of neuramin-lactose from bovine colostrum

Because of the interest in neuraminidase and its relation to infection by the influenza group of viruses, there is a need for a convenient, standard substrate for this enzyme. N-L, discovered by TRUCCO AND CAPUTTO[1,2], has been shown to be such a substrate[3-6]. Several methods for the preparation of this material have evolved which utilize ion-exchange resins, in some cases also making use of activated carbon[2,4,7]. However, these procedures all involve problems that make it difficult to produce pure N-L easily and quickly without hydrolyzing it. A need for a method that satisfied these conditions was recognized when one of us (L.W.M.) required substantial amounts of this compound for kinetic studies on the solubilized viral neuraminidase[8]. Such a process is hereby reported and involves only the following steps. The dialysate of fresh bovine colostrum was passed through a Dowex-1 column and the N-L was eluted with sodium chloride. This eluate was passed through an activated-carbon column and the desired material eluted with 60 % ethanol. Lyophilization then followed after removal of the alcohol by vacuum evaporation.

500–1000 ml fresh or frozen bovine colostrum (obtained less than 1 day after parturition) was dialyzed against 5 volumes distilled water for 24 h at 4°, using a magnetic stirrer and Visking dialyzing tubing (23/32″). The outer water was changed and the dialysis allowed to proceed for another 24 h. The volume of the combined dialysates was reduced to 100–200 ml on a Rinco flash evaporator at a temperature less than 50°.

A 46.0 × 6.6 cm column of Dowex-1 resin, 200–400 mesh, was prepared by washing the resin successively with 2 column volumes each of 3 *N* HCl, 3 *N* NaOH and 3 *N* HCl, and then washed with water until the wash fluid was neutral and free of chloride ion as determined with $AgNO_3$. The colostrum dialysate was poured onto the column and washed through with 2 column volumes of distilled water. No neuraminic acid-containing compounds came through in the wash fluid, using this size column and up to 1 l colostrum, as determined by the method of WERNER AND ODIN[10] using Ehrlich's reagent, but much sugar was present in the eluate as measured by a modification* of the orcinol–H_2SO_4 procedure[11]. Then 3–4 column volumes of 0.1 *M* NaCl were passed through the column followed by the same amount of 1.0 *M* NaCl. All eluates were tested for sialic acid (the general name for neuraminic acid and derivatives) and for hexose (Fig. 1).

The fraction eluted with 0.1 *M* NaCl consistently contained a hexose: sialic acid molar ratio of 2:1 with little or no free sialic acid present as shown by the thiobarbituric acid procedure of WARREN[12]. This fraction was then passed through an activated-carbon column (Darco-60), consisting of an approx. 2-cm layer of filter-aid, over sintered glass, covered with 21.5 cm of a mixture of filter-aid and Darco-60 (2:3, wet volume) previously washed with HCl, NaOH and absolute ethanol. The layer of activated carbon was overlaid with another cm of filter-aid, and the column was also prewashed with 60 % ethanol. (The 60 % ethanol wash is absolutely necessary because at that concentration of alcohol a small amount of black soluble material is washed off the column. Once this material is removed, the column can be used over and over without any further appearance of this material and without any further

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Abbreviation: N-L, neuramin-lactose.

* 0.5 ml of sample plus 0.5 ml 1.6 % orcinol (recrystallized from benzene) plus 3.0 ml of 70 % H_2SO_4; mix well and heat in an 80° water bath for 15 min; cool and read at 540 mμ.

washing except regeneration with water.) After the addition of sample, the column was washed with distilled water until the eluate was free of NaCl. This generally required 1–2 column volumes water. Then, 5 column volumes 60 % ethanol were added to elute the adsorbed N-L. Usually, these eluate tubes were checked for their concentration of hexose and sialic acid (Fig. 2). All tubes containing N-L were combined and the alcohol removed by evaporation under reduced pressure (Rinco flash evaporator). The neutral, colorless water solution of N-L was lyophilized, yielding a white, hygroscopic solid, which was 95–100 % pure according to the amount of hexose and sialic acid present. The yield could be as much as 0.56 g/l colostrum. Chromatography with the methyl ethyl ketone–acetone–water–formic acid (3:1:1:0.1) solvent system of HOGSTROM[9] revealed no additional spots when sprayed with aniline oxalate for reducing sugars, resorcinol reagent for sialic acid[13] or ninhydrin (0.4 % in acetone). Treatment with viral neuraminidase followed by chromatography yielded two spots corresponding to lactose and sodium N-acetyl neuraminate.

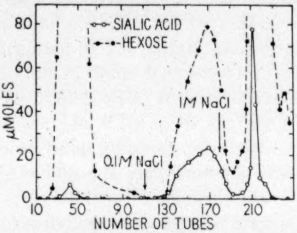

Fig. 1. Separation of neuraminic acid constituents of bovine colostrum dialysate on a Dowex-1-X[4] column, Cl$^-$ form, 200–400 mesh, using solutions of 0.1 *M* and 1.0 *M* NaCl as the eluting agents.

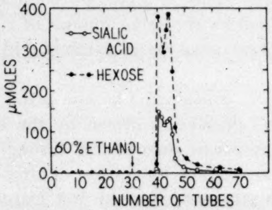

Fig. 2. Adsorption on Darco-60–filter-aid column and elution with 60 % ethanol of the fraction eluted with 0.1 *M* NaCl off the Dowex-1 column.

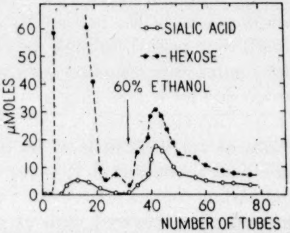

Fig. 3. Removal of salt and contaminating carbohydrate from the sialic acid peak eluted with 1.0 *M* NaCl by adsorption on a Darco-60–filter-aid column and elution with 60 % ethanol.

The eluates which followed the addition of 1.0 *M* NaCl to the Dowex column proved to form several hexose-containing peaks but only one which contained neuraminic acid as well as much hexose. The fractions contained in this peak were combined and run through the Darco-60–filter-aid column under the same conditions as before.

After all the salt had been washed through the column, the 60% ethanol was added to elute the desired material, temporarily termed neuramin-X (N-X).

Upon analysis it was found that much hexose-containing material had been washed away with the salt (Fig. 3). The eluates containing the sialic acid material were combined, evaporated under reduced pressure to remove the alcohol, and lyophilized, yielding a white hygroscopic solid. Analysis of this material showed it to contain sialic acid, glucose and galactose, but no hexosamine. It contains components which give a reaction with ninhydrin after acid hydrolysis and chromatography, and it does not appear to be susceptible to neuraminidase action. It absorbs strongly in the u.v., showing a peak at $262\ m\mu$; chromatography with the above solvent system reveals three spots which absorb strongly in the u.v. One of these spots reacts to stains for reducing sugar, sialic acid, phosphate and amino acids (ninhydrin). Further studies on this material are now in progress.

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Concentration of unbound amino acids in human platelets

The presence of unbound amino acids in platelets has been noted by several workers[1–3]. To our knowledge amino acid concentrations in the platelets have not been determined quantitatively. The results of five analyses are reported herein.

The platelets were supplied by the Protein Foundation, Jamaica Plain, Massachusetts. They were separated from blood collected in acid citrate–dextrose solution by the ADL-Cohn Fractionator (A. D. Little and Company, Cambridge, Massachusetts) by the method of TULLIS *et al.*[4]. The platelets were washed with two 25-ml portions of a 1% mildly acetylated albumin in $0.15\ M$ NaCl solution, then eluted from the fractionator bowl with 5 ml of the same solution. (The albumin was acetylated to prevent its binding of tryptophan, Expt. 135 DI, Table I, ref. 5.) Aliquots of the platelet suspension were taken for platelet counts and for measurements of packed platelet volume. (The latter were conducted in microhematocrit tubes at a centrifugal force of 5,000 × g for 20 min.) Leukocyte contamination was apparently 1 per 10,000 platelets. The volume of the remaining solution was measured and then made up to a total of 10 ml with water. The suspended platelet solution was twice frozen to lyse the platelets and dialyzed by the thin-layer technique[6] (using Visking tubing size 23/32) against 40 ml water for 2 h. The dialysate was dried from the frozen state. The dried residue from the dialysate was dissolved in Tergitol-Safranin dye solution[7] in the ratio of 0.43 ml/ml of the packed platelet volume dialyzed. Analyses were conducted by paper chromatography[7]. To express the concentrations of the amino acids on a kg of water basis the platelets were taken to be 81 % water[8].

The mean concentrations of 22 substances in platelets and the ratio which these concentrations bear to the normal plasma concentrations are listed in Table I. For comparison the ratio of the leukocyte-plasma concentrations of the unbound amino acids in normal human blood[7] are also given. The concentrations of glutamic acid, taurine, O-phosphoethanolamine and proline were approximately the same in the platelets (3,600, 23,800, 2,300 and 600 μmoles/kg water respectively) as in normal leukocytes (2,745, 28,683, 2,651 and 862 μmoles/kg water respectively)[7]. With

TABLE I

UNBOUND AMINO ACID CONCENTRATIONS IN PLATELETS

	Number of subjects	*μmoles/kg water*	*Platelet-plasma ratio**	*Leukocyte plasma ratio***
Alanine	5	1,160 ± 69***	2.9	7.5
α-Amino-n-butyric acid	4	120 ± 24	4.2	—
Arginine	4	120 ± 14	1.7	4.2
Glutamic acid	5	3,600 ± 895	high	high
Glutamine	5	850 ± 196	1.7	5.2
Histidine	5	300 ± 155	3.3	9.8
Lysine	5	410 ± 127	2.0	10.2
Methionine	4	160 ± 25	7.4	14.5
Ornithine	5	870 ± 311	10.2	20.6
Phenylalanine	4	270 ± 30	5.0	13.6
Proline	5	600 ± 54	3.2	4.8
Threonine	5	930 ± 50	5.6	14.4
Tryptophan	4	100 ± 18	5.5	11.4
Tyrosine	5	280 ± 23	4.0	12.0
Valine	5	550 ± 16	2.7	6.6
Ergothioneine	5	50		
Ethanolamine	5	350		
Leucine plus isoleucine	5	920 ± 81	4.5	10.4
Serine plus glycine	5	5,000 ± 1,460	8.2	22.1
Taurine	5	22,800 ± 940	high	high
Urea	4	4,900 ± 780	0.9	—
O-phosphoethanolamine	5	2,300 ± 460	high	high
Serotonin	3	140 ± 80 §	high	—
Platelet volume	5	0.014 ±0.0007	—	—

* The platelet–plasma ratio was computed using the average plasma concentrations found for 15 normal fasting subjects[7].

** Taken from ref. 7.

*** Standard deviation of the mean.

§ Serotonin creatinine sulfate was added to the standard solution for this estimation.

the exception of these four substances and lysine, whose concentration was one-fifth the concentration found in the leukocytes, the concentrations of the remaining compounds were one-third to one-half the leukocyte values. The concentrations of the basic amino acids, histidine, lysine and ornithine varied more in the analyses than the other amino acids.

The mean serotonin concentration found in the platelets was 140 μmoles/kg water. (The R_F value of serotonin identified with EHRLICH's reagent was approximately that of leucine.) On the basis of a platelet count of 300,000/mm^3 whole blood this value, *i.e.*, 140 μmoles/kg platelet water, was slightly less than the value 220 ± 80 (0.16 ± 0.06 μg/ml blood) reported by HARDISTY AND STACEY[9] or the range of values 140–400 (0.1–0.3 μg/ml blood) reported by UDENFRIEND, WEISBACK AND CLARK[10] for normal human subjects.

A prominent unknown zone, positive to the PtI_4 reagent but negative to ninhydrin, with an R_F value of approximately that of alanine in Solvent A was found in all platelet analyses. Treatment of the zone with H_2O_2 destroyed its response to the PtI_4 test. The substance was not otherwise identified. Traces of this zone have occasionally been observed in leukocyte analysis, but not in plasma or red-cell analyses[7].

Amino acid values obtained from platelets which had been packed by centrifugation, but not washed, were similar to those reported in Table I when compared on a platelet basis.

The average packed platelet volume 0.014 ml/10^9 platelets, which was obtained by using a centrifugal force of 5,000 × g, compares favorably with the value of 0.017 ml/10^9 platelets obtained by HARDISTY AND STACEY[9] in which a centrifugal force of 2,000 × g was used.

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Preliminary Notes

Protéines anormales de phage synthétisées en présence de ribonucléase

Les particules de phage produites par un *Bacillus megaterium* lysogène après induction sont dépourvues de caractère infectieux dans leur immense majorité si de la ribonucléase de boeuf a été ajoutée à la culture au moment où débute la synthèse des protéines virales[1]. Il semble que la majorité, sinon la totalité des protéines virales synthétisées dans ces conditions aient une structure anormale.

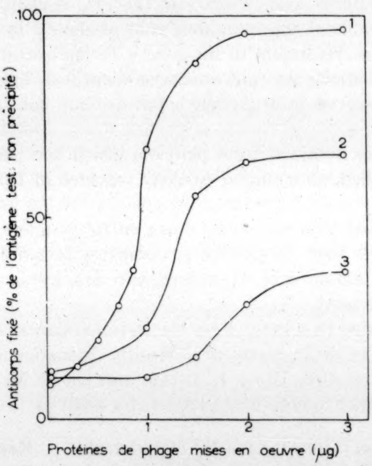

Fig. 1. Quantités d'anticorps fixées lorsque des quantités croissantes de protéines de phages normaux (I) ou de protéines de phages cultivés en présence de ribonucléase [(2): 60 μg/ml; (3): 100 μg/ml] sont ajoutées à des quantités constantes de sérum anti-phage normal. La quantité d'anticorps fixée est supposée proportionnelle au % non précipitable d'une quantité test de protéines de phages normaux ajoutée après la fin de la réaction étudiée.

Les constituants protéiques des phages cultivés en présence de ribonucléase et dispersés par l'action d'un oscillateur Raytheon 10 KC ne fixent en effet qu'une partie des anticorps précipitants d'un sérum anti-phage normal (Fig. 1), et ne sont précipités que très partiellement par ce sérum (Fig. 2).

D'autre part, les protéines virales édifiées en présence de ribonucléase, et restées libres au moment de la lyse des cellules bactériennes par le lysozyme, apparaissent dans le diagramme d'élution d'une chromatographie sur colonne de DEAE-cellulose sous l'aspect d'une large bande, correspondant vraisemblablement à de nombreuses espèces protéiques non séparées. Cette fraction complexe occupe toujours la même position dans le diagramme quel que soit le temps écoulé entre le début de la synthèse

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Abbréviation: DEAE-cellulose: diethylaminoethyl-cellulose.

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des protéines virales et le prélèvement des cellules. Dans le cas des cultures témoins le diagramme d'élution montre au contraire un petit nombre de pics étroits, largement séparés. Les divers pics observés apparaissent à des moments différents de l'évolution du sytème (Fig. 3).

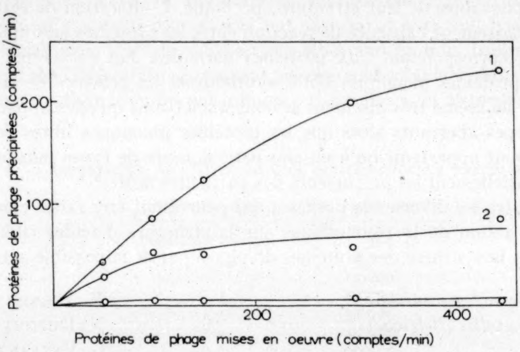

Fig. 2. Quantités de protéines normales de phage (I) et de protéines synthétisées en présence de ribonucléase [(2) : 40 µg/ml; (3) : 60 µg/ml] qui précipitent après adjonction de quantités croissantes de chacun des antigènes testés à une quantité fixe de sérum antiphage normal. Les quantités d'antigènes sont exprimées en comptes/min; les diverses préparations étaient marquées par du ^{35}S à la même radioactivité spécifique.

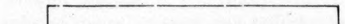

Fig. 3. Protéines phagiques libres, précipitables par du sérum anti-phage normal, recueillies après chromatographie sur colonne de DEAE-cellulose d'un lysat par le lysozyme de cellules de *B. megaterium* induites. Cellules lysées par le lysozyme 15 min avant le début de la lyse spontanée. Particules de phage éliminées par centrifugation. Elution à l'aide d'un tampon phosphate 0.02 *M*, pH 7.4. Fractions de 1 ml. Cultures faites en l'absence de ribonucléase (1) ou en présence de 60 µg/ml de l'enzyme (2).

Ces divers effects de la ribonucléase ne se manifestent pas si le site enzymatiquement actif est préalablement bloqué par carboxyméthylation d'une histidine suivant la méthode de BARNARD ET STEIN[2]. Il paraît donc probable que la ribonucléase agit sur la structure d'acides ribonucléiques intervenant dans la synthèse des protéines de phage et responsables de leur structure spécifique. L'altération de cette dernière expliquerait parfaitement l'absence de réaction entre les protéines anormales formées et les anticorps correspondant aux protéines normales. Les caractères chromatographiques des protéines phagiques libres synthétisées en présence de ribonucléase s'expliqueraient de même très aisément si nous admettions qu'elles appartiennent à de nombreux types aberrants alors que les protéines phagiques libres des cultures témoins ne peuvent appartenir qu'à un très petit nombre de types puisqu'elles sont sans doute essentiellement les précurseurs des particules mûres[3,4].

Un choix entre les diverses hypothèses qui pourraient être faites concernant le mécanisme de l'action de la ribonucléase sur la structure d'acides ribonucléiques intervenant dans la synthèse des protéines de phage paraît impossible actuellement.

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Bacteriocidal action of mitomycin C

The results of several investigations[1,2] have shown that the anti-tumour antibiotic, Mitomycin C, possesses antibacterial and radiomimetic properties. Moreover, in susceptible microorganisms it inhibits the biosynthesis of DNA[3]. The action of Mitomycin suggested a possible relationship to the phenomenon of "thymineless death[4]", a characteristic potentially useful for the selection of biochemical mutants in bacterial populations[5]. We have therefore examined its effect on several strains of *Escherichia coli*. The results show that Mitomycin C causes these bacteria to break down their DNA rapidly, acid-soluble products being formed.

A 12-h culture of *E. coli* (strain $15T^-$), grown with shaking at 37°, was diluted into 5 vol. glycerol–ammonium-salts medium supplemented with thymidine (30 mg/l). The bacteria were harvested by centrifugation while in the logarithmic phase of growth, washed thrice with thymidine-free medium, and resuspended in medium containing $[^3H]$thymidine. Following 90-min incubation under conditions which prevented the occurrence of "thymineless death", the culture was once again centrifuged, the bacterial pellet washed free of radioactive thymidine, resuspended and incubated in fresh medium containing graded concentrations of Mitomycin C. After suitable intervals of time, the cultures were acidified with cold dilute $HClO_4$ and centrifuged. The supernatant solutions were neutralized with KOH, and the salts

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Abbreviations: DNA, deoxyribonucleic acid.

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which precipitated in the cold were removed by centrifugation. The radioactivity of the extracts was determined in a windowless gas-flow counter.

The results obtained in such an experiment are given in Table I. It is seen that cultures treated with increasing concentrations of Mitomycin C yielded a substantial proportion of their DNA in the form of acid-soluble degradation products. The accumulation of these substances was not associated with cell lysis, and occurred with cells which were actively synthesizing RNA and protein. In some experiments, up to 63 % of the DNA originally present became acid-soluble within 2 h. The presence of thymidine (or thymine) did not influence these effects of Mitomycin treatment.

TABLE I

APPEARANCE OF [^{3}H]THYMIDINE IN ACID-SOLUBLE FRACTION

Mitomycin (μg/ml)	*Extract (counts/min)**
Zero-time control	989
0	1,710
0.5	12,200
1.0	16,000
2.0	31,000
5.0	47,500
10.0	66,600
20.0	74,300

* Corrected for self-absorption. Samples incubated for 2.5 h, 37° (see text).

The rate of breakdown, which varied with bacterial population density as well as with the concentration of Mitomycin C, was linear with time for periods up to 2.5 h (Fig. 1). Chromatography of the extracts of such cultures has shown that about 95 % of the radioactivity was in thymine; the remainder was present as thymidine and a nucleotide which was not thymidine 5′-phosphate.

This action of Mitomycin C has been found to require the presence of Mg^{++} and to be inhibited by streptomycin, as in the breakdown of the DNA of superinfecting bacteriophages[7]. Following a brief exposure, the presence of the antibiotic is not required for a maximal rate of DNA breakdown to persist. Experiments have been performed to compare the rates of DNA synthesis and breakdown over a range of concentrations of Mitomycin C, these processes being measured respectively by uptake into and release from the acid-insoluble fraction of radioactive precursors or breakdown products. The resultant data are consistent with the assumption that the fraction of a bacterial population which is affected by Mitomycin and depolymerizing its DNA does not incorporate precursors into DNA, and, conversely, that the remaining fraction, which is capable of incorporating thymidine into DNA, does not release acid-soluble fragments into the medium. It should be noted that bacteria which have undergone "thymineless death" do not degrade their DNA, and that this effect of Mitomycin C is therefore not merely the result of inhibition of DNA synthesis.

Examination by phase[6] and electron microscopy of bacteria treated with Mitomycin has made possible the clear visualization of disintegrating nuclear bodies. These and other morphological, genetic, and biochemical findings form the subject of a full-length communication which is being submitted for publication to this journal. They suggest that the primary action of Mitomycin C leads to a scission of the DNA strand in susceptible bacteria; such DNA would no longer be capable of replication, and the observed inhibition of synthesis follows. This formulation would appear to explain satisfactorily the irreversible bacteriocidal action of Mitomycin C.

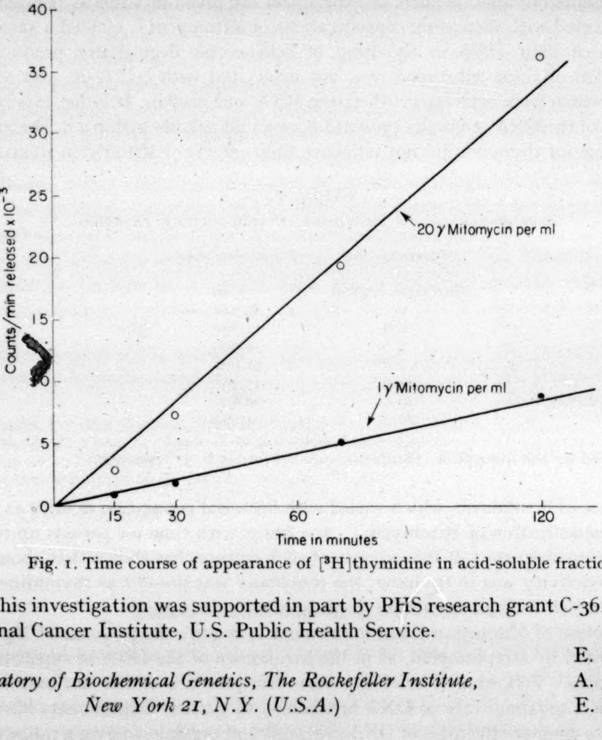

Fig. 1. Time course of appearance of [^{3}H]thymidine in acid-soluble fraction.

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Thymine starvation and enzyme synthesis

Cultures of *E. coli* strain 15_{T-} suffer progressive and roughly parallel losses of viability and capacity to form the inducible enzyme β-galactosidase after about one generation

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Abbreviations: RNA, ribonucleic acid; TMG, methyl-β-O-thiogalactopyranoside.

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of growth in thymineless medium[1,2]. Net syntheses of protein and RNA are somewhat less sensitive to thymine starvation[3]. These findings would indicate that when a cell is rendered incapable of replication it also becomes incapable of synthesizing specific proteins. We have recently obtained a divergent result for the enzyme alkaline phosphatase, which suggested another possibility.

The effects of thymine starvation on phosphatase[4], β-galactosidase[5], β-galactosidase permease[6], and protein[7] formation and on viability are presented in Fig. 1.

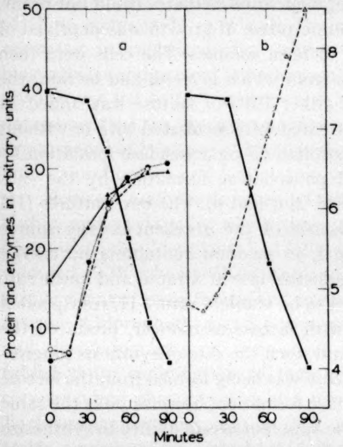

Fig. 1a. Formation of β-galactosidase, β-galactosidase permease, and protein, and decline in viability during thymine starvation. The medium utilized in all experiments was the synthetic glycerol–casamino acids H medium, supplemented with thymine, which has been described previously[10]. Such alterations as were made are as noted below. The inducer for the β-galactosidase enzymes was $5 \cdot 10^{-3}$ *M* TMG. The arbitrary unit of β-galactosidase (●—●) = 0.275 enzyme units/ml cells[5], as measured at 37°. The arbitrary unit of β-galactosidase permease (○—○) = accumulation of 2 counts/min/ml cells during 15 min incubation from a suspension containing $3 \cdot 10^4$ counts/min/ml radioactive TMG ($5 \cdot 10^{-3}$ *M*). The arbitrary units of protein (▲—▲) = 12 μg protein/ml. ■—■, viability. Fig. 1b. Formation of alkaline phosphatase and decline in viability during thymine and phosphorus starvation. The arbitrary unit of phosphatase (△—△) = 13.3 enzyme units/ml cells[4].

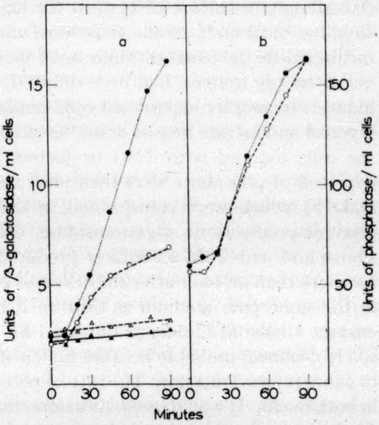

Fig. 2a. Formation of β-galactosidase by thymine-starved cells. The cells (about $3 \cdot 10^7$/ml) had been incubated for 60 min without thymine; the residual viability was then 5%. The cells were resuspended at the same density in medium devoid of an energy source (Cultures I and II, ●—●, ○—○), or in medium containing glycerol and lactate (Cultures III and IV, ▲—▲, △—△). Cultures I and III were induced with $5 \cdot 10^{-3}$ *M* TMG, Cultures II and IV with 0.1 % lactose. Fig. 2b. Formation of phosphatase by thymine-starved cells. The cells (about $3 \cdot 10^7$/ml), were incubated without thymine for 60 min, then resuspended at the same density in medium devoid of an energy source (Culture I, ●—●), or in medium containing glycerol and lactate (Culture II, ○—○).

The phosphate starvation required for phosphatase synthesis clearly did not protect the cells from thymineless death. It is apparent that the synthesis of phosphatase, but not that of the β-galactosidase and the permease, progressed well beyond an hour of thymine starvation, at which time the viability of the cultures had declined to a negligible figure.

It will be noted that at the point at which β-galactosidase synthesis has virtually ceased the rate of protein synthesis has diminished considerably. This suggested that decreased anabolic activity of the cells might have led to an accumulation of the products of catabolic reactions within the cells. As previously discussed, these products would repress the formation of an enzyme (in this case β-galactosidase) whose activity would augment their already large metabolic pools (glucose effect)[8,9].

To test this hypothesis it was necessary to place thymine-starved cells under conditions in which this overproduction of metabolic intermediates could not occur. Accordingly, a culture of 15_{T^-} in the logarithmic phase of growth was deprived of thymine until 90 % of the cells were unable to form colonies. The cells were then incubated in the usual medium or in medium from which glycerol and lactate, the major energy sources, had been omitted, and either TMG or lactose was added as inducer. In another experiment cells similarly treated were incubated with or without glycerol and lactate in a medium devoid of phosphate. β-Galactosidase formation by the cells induced with TMG or lactose, and phosphatase formation by the cells deprived of phosphate were then measured (Fig. 2, a and b). The two cultures (III and IV) which were resuspended in the presence of the excellent energy source glycerol produced no β-galactosidase. Culture I, in medium containing no energy source and with TMG as inducer produced β-galactosidase at a rapid and linear rate for more than an hour after the cells had ceased to be viable. Culture II, resuspended in the same poor medium as Culture I, but with lactose as inducer, produced the enzyme almost as rapidly as Culture I for 30 min, then the rate of synthesis progressively declined, probably because sufficient glucose was being formed from the lactose to cause repression again. The rate of phosphatase formation, however, was the same in both media. It would seem, therefore, that the apparent loss of ability to synthesize β-galactosidase which has been seen to coincide with thymineless death is not an irreversible result of nuclear damage, but is due to repression. It is conceivable that an analogous situation may have existed in cultures being progressively inactivated by ^{32}P decay, in which capacity for synthesis of specific enzymes was found to decline at about the same rate as viability[10]. These experiments will be repeated with induction performed in the absence of energy source.

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Chlorophyll biosynthesis in wheat leaves

The suggestion[1] that the pathway of biosynthesis of the dihydroporphyrins in higher plants is similar to the known pathway for the synthesis of porphyrins in animals[2] has been supported by the experiments of SALOMAN *et al.*[3], DELLA ROSA *et al.*[4], BRZESKI AND RÜCKER[5], and SHLYK *et al.*[6]. WANG *et al.*[7], however, having failed to detect any radioactivity in the chlorophylls of mature excised primary leaves of wheat following administration of [2-^{14}C]glycine suggest that glycine is a poor precursor of these compounds in wheat. During investigations of the biochemical changes occurring in cold-hardening winter-wheat plants, we have studied chlorophyll biosynthesis and present here the results of preliminary experiments in which [2-^{14}C]-glycine was fed to both mature and immature intact primary leaves of wheat. The data show that the apparent inefficiency of glycine as a chlorophyll precursor in the experiments of WANG *et al.*[7] was probably a reflection of a very low turnover rate of both chlorophylls *a* and *b* in mature wheat leaves.

Triticum aestivum L. emend Thell. var. Kharkov 22 M.C. was grown at 21° for both 5 and 13 days by which times the young plants had still-expanding primary leaves while the older plants had fully-expanded primary leaves. The illuminated plants were fed [2-^{14}C]glycine (New England Nuclear Corp.) through the roots for 4 h. The primary leaves were frozen and ground in liquid nitrogen and extracted with cold acetone. Chlorophylls *a* and *b* were purified from the extracts by solvent partition and chromatography on icing-sugar columns. The purity of the products was established by examination of their absorption spectra[8] and by conversion to the corresponding pheophytins which were purified by chromatography on icing sugar. To determine the incorporation of ^{14}C into phytol, the chlorophylls were also degraded to the corresponding pheophorbides[8], which were freed of unreacted pheophytins by partition between 1 % NaOH and diethyl ether[10].

The molar extinction coefficients of ZSCHEILE AND COMAR[8] were used in determining chlorophylls *a* and *b* and pheophytins *a* and *b*. The same extinction coefficients were used for the pheophorbides as for the corresponding pheophytins[11]. Radioactivity determinations (to within 2 % standard error) were made on infinitely thin layers on glass planchets in a windowless, methane, gas-flow counter operating in the proportional region at an absolute efficiency of 50 %.

TABLE I

INCORPORATION OF ^{14}C FROM [2-^{14}C]GLYCINE ($1.72 \cdot 10^{9}$ DISINTEGRATIONS/MIN/MG CARBON) INTO THE DIHYDROPORPHYRINS OF 5- AND 13-DAY-OLD PRIMARY LEAVES OF KHARKOV 22 M.C. WHEAT

Compound	*Specific activity**	
	5-day-old	*13-day old*
Chlorophyll *a*	6.52	0**
Pheophytin *a*	5.21	0
Pheophorbide *a*	5.75	–
Chlorophyll *b*	4.54	0***
Pheophytin *b*	1.84	0
Pheophorbide *b*	2.28	–

* In disintegrations/min/mg carbon × 10^{-5}.
** Less than 160 disintegrations/min/mg carbon
*** Less than 640 disintegrations/min/mg carbon.

The data in Table I show that mature (13-day) primary leaves synthesized virtually no chlorophyll in 4 h. The residue from the acetone extract of these leaves contained several times as much ^{14}C as that from the younger leaves. The low dilution of the ^{14}C in the chlorophylls of the young (5-day) leaves indicates that the methylene carbon of glycine is an efficient precursor of both chlorophylls *a* and *b*, and the similarities between the specific activities of the corresponding pheophytins and pheophorbides indicate that this carbon is also a precursor of phytol in wheat leaves. The loss of radioactivity on conversion of the chlorophyll *b* to pheophytin *b* shows that the former compound was impure. The loss during the corresponding conversion of chlorophyll *a* is probably within the experimental error.

Our data also suggest that it is highly improbable that chlorophyll *a* is formed from chlorophyll *b*, but the opposite possibility is not ruled out. Further experiments are in progress.

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N-Methyladrenaline, a new catecholamine in the adrenal gland

In a study on the properties of the enzyme that converts noradrenaline to adrenaline, it was observed that the incubation of the soluble supernatant fraction obtained from beef adrenal glands with adrenaline and [^{14}C-*Me*]-S-adenosylmethionine formed a compound that had the same R_F values as N-methyladrenaline. This led to an investigation of the normal occurrence of N-methyladrenaline in the adrenal gland.

Frozen adrenal glands from cows were homogenized with 5 vol. 0.4 N $HClO_4$ in a Waring blender and the resulting suspension was centrifuged. To the supernatant solution was added 0.1 vol. each of 1 % disodium ethylenediamine and 1 % freshly prepared ascorbic acid and the pH carefully adjusted to 8.4 with 0.5 N K_2CO_3. After centrifugation the clear supernatant fluid was passed over a column of aluminium

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oxide (Woelm, neutral) and the catechols absorbed and eluted as described by Weil-Malherbe and Bone[1]. The eluates were evaporated to a small volume *in vacuo* and an aliquot subjected to two-dimensional paper chromatography (ascending) with Whatman No. 1 filter paper, using butanol–acetic acid–water (4:1:1) as the first solvent system, and phenol–water (0.85:0.15) saturated with SO_2 as the second. After the paper was washed several times with benzene and dried, it was sprayed first with 0.44% $K_3Fe(CN)_6$ in phosphate buffer, pH 7.8, and then with ferric sulfate[2]. One of the blue spots appearing had the same R_F values (0.40, 0.72) as authentic N-methyladrenaline.

Additional evidence for the identity of N-methyladrenaline was obtained by forming an O-methyl derivative. The concentrated eluate from the alumina column was distributed along a starting line of Whatman No. 1 filter paper and subjected to ascending chromatography using the phenol–water–SO_2 solvent system. The paper was washed as described above and a strip of an area corresponding in R_F value of 0.60 to 0.80 was cut out and a 3-O-methyl derivative made enzymically with catechol-O-methyl transferase[3] as follows: The paper was cut into small pieces and added to a beaker containing a soluble supernatant fraction obtained from 500 mg rat liver, 0.2 ml 0.5 M $MgCl_2$, 100 mμmoles [^{14}C-*Me*]-S-adenosylmethionine (46,000 counts/min) and 2 ml 0.5 M phosphate buffer, pH 7.9. After 2-h incubation, the reaction mixture was adjusted to pH 10.0 with NaOH and borate buffer and extracted twice with 5 vol. of a mixture containing toluene–isoamyl alcohol (3:2). The organic phase was evaporated to a small volume and co-chromatographed with synthetic N-methylmetanephrine (N-methyl-3-O-methyladrenaline) in 4 solvent systems. Radioactive peaks were found that coincided in R_F values with authentic N-methylmetanephrine. The following were the solvent systems used and the R_F values obtained; butanol–acetic acid–water (4:1:1), R_F 0.68; isopropanol–ammonia (8:1), R_F 0.90; butanol–methanol–ammonia (8:1:1), R_F 0.81; butanol–ethanol–water (4:1:1), R_F 0.46. An unidentified radioactive peak whose R_F values differed from those of N-methylmetanephrine was also present.

A compound having the same R_F values as N-methyladrenaline was also found in the adrenal glands of the monkey, rat, rabbit and guinea pig.

When N-methyladrenaline was administered intravenously to a rat, large quantities of N-methylmetanephrine (free and conjugated) were found in the urine.

From the observations described above, the following scheme for the formation and metabolism of N-methyladrenaline is suggested:

Adrenaline ⟶ N-methyladrenaline ⟶ N-methylmetanephrine

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Enzymic synthesis of bacterial phytoene from [2-^{14}C]mevalonic acid

A mutant of *Staphylococcus aureus*, which lacks the biosynthetic ability to produce bright-colored pigments, was separated and was found to accumulate the carotenoid phytoene[1]. The biosynthesis of phytoene by enzyme preparations from this mutant has been studied with [2-^{14}C]mevalonic acid.

The coccus was cultivated for 18 h at 34° in a nutrient broth containing 2% glycerol (pH 7.5) with continuous shaking. The resulting cells were collected by centrifugation at 4,000 rev./min for 10 min and were washed with 0.3% aq. NaCl.

After grinding the cells with alumina for 10 min under cooling, enzymes were extracted with 0.01 *M* Tris buffer (pH 8.0). The supernatant obtained after the removal of alumina and cell debris by centrifugation (5,000 rev./min for 10 min and 8,000 rev./min for 10 min) was used as the enzyme preparation for this study.

The incubation was carried out aerobically at 37° for 3 h. At the end of this period, 2 ml 10% ethanolic NaOH, 10 mg *p*-hydroquinone and 1.00 ml of a solution in petroleum ether of pure bacterial phytoene of absorbancy 8.500 at 286 mµ were added. The mixture was heated for 15 min in a water bath under N_2 with gentle reflux. After cooling, 2 ml water and 5 ml petroleum ether were added and after vigorous shaking the unsaponifiable matter was extracted into the petroleum ether layer. The petroleum ether extract was washed three times with water and dehydrated with anhyd. Na_2SO_4. The aliquot of the unsaponifiable fraction thus obtained was dried on alumina planchets and the radioactivity was counted as an infinitely thin sample in a gas-flow counter.

Separation of the carotenoid was carried out chromatographically on an activated-alumina column (150–200 mesh). The dry petroleum ether solution containing the unsaponifiable fraction was poured on the column of alumina (1 cm × 15 cm). After washing the column with petroleum ether, the carotenoid adsorbed was eluted with petroleum ether–ether (98:2). The ultraviolet-absorption spectrum of each 3 ml of the eluate was measured by a recording spectrophotometer. There was at least

TABLE I

EFFECT OF FLUORIDE, NICOTINAMIDE AND/OR EDTA UPON CONVERSION OF DL-[^{14}C]MEVALONIC ACID INTO UNSAPONIFIABLE FRACTION AND BACTERIAL PHYTOENE

Each system contained Tris buffer (pH 8.0), 40 µmoles; ATP, 30 µmoles; DPN, 2 mg; TPN, 0.5 mg; 6-phosphogluconate, 2 µmoles; enzyme solution, 0.3 ml (protein concentration, 52 mg/ml); DL-[2-^{14}C] mevalonic acid, 1.45 µg (10,000 counts/min); $MgCl_2$, 10 µmoles; $MnSO_4$, 4 µmoles, and (where indicated) EDTA, 7 µmoles, nicotinamide, 50 µmoles and/or KF, 30 µmoles, in a total volume of 0.6 ml. Incubation in air for 3 h at 37°.

Addition	*Unsaponifiable fraction (counts/min)*	*Bacterial phytoene (counts/min)*
None	2115	495
KF	800	435
KF, EDTA	125	115
KF, nicotinamide	1540	705
KF, nicotinamide, EDTA	125	30

Abbreviations: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

90 % recovery of the phytoene. Samples (1.00 ml) of the two eluates containing the most phytoene were dried on alumina planchets and the radioactivity counted as before.

TABLE II

EFFECT OF COFACTORS ON THE BIOSYNTHESIS OF BACTERIAL PHYTOENE

Complete system contained Tris buffer (pH 8.0), 40 μmoles; ATP, 30 μmoles; DPN, 2 mg; TPN, 0.5 mg; 6-phosphogluconate, 2 μmoles; enzyme solution, 0.3 ml (protein concentration, 63 mg/ml); DL-[2-^{14}C]mevalonic acid, 1.45 μg (10,000 counts/min); $MgCl_2$, 10 μmoles; $MnSO_4$, 4 μmoles; nicotinamide, 50 μmoles; KF, 30 μmoles; and (where indicated) EDTA, 7 μmoles and bacterial nucleotide*, 10 mg in a total volume of 0.6 ml. Incubation in air at 37° for 3 h.

	(counts/min)	
Cofactors	*Unsaponifiable fraction (counts/min)*	*Bacterial phytoene (counts/min)*
Complete system	1433	1195
— ATP	262	149
— TPN	1895	1029
— TPN, 6-phosphogluconate	1098	935
— TPN, DPN	1044	711
— Mg, Mn + EDTA	454	83
+ Bacterial nucleotide	1892	1398

* Bacterial nucleotide was prepared from the boiled extract of cells by acetone precipitation and dried in a vacuum desiccator.

As shown in Table I the addition of nicotinamide favored the biosynthesis of bacterial phytoene. One reason may be the prevention of the breakdown of pyridine nucleotides[2]. The cofactor requirements are shown in Table II. ATP, TPNH and metals (Mg, Mn) are apparently essential as in the case of squalene[3–5]. There remains a possibility of the participation of other cofactor(s) in this system, judging from the stimulatory effect by the bacterial nucleotide.

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Search for a dihydroxy acid dehydrase in rat and microbial tissues[*]

Valine can be replaced by its α-keto analogue for the growth of the weanling rat[1,2]. This nutritional observation is generally interpreted as a conversion of the keto acid to the amino acid due to tissue transaminases.

The last steps in the biosynthesis *de novo* of valine in *Neurospora crassa*[3,4], *Escherichia coli*[3,4], *Torulopsis utilis*[5] and *Saccharomyces cerevisiae*[6] involves the dehydration of α,β-dihydroxyisovaleric acid to α-ketoisovaleric acid and its transamination to yield valine. Thus it is desirable to conduct experiments to test for the presence of the next to the last enzymic step in the rat.

A valine-free basal diet was obtained by using a mixture of 18 crystalline amino acids[7] in a synthetic diet. The male weanling rats (four per group) on the basal diet steadily lost weight during the 2-week experimental period (Table I). Supplementation of the basal diet with valine produced growth. Addition of an equimolar amount of DL-α,β-dihydroxyisovaleric acid failed to stimulate growth. As observed by previous investigators[1,2], α-ketoisovaleric acid supported growth in the rats. Though this growth data is in accord with the presence of transaminase, it also suggests the absence of the dihydroxy acid dehydrase in the rat.

In addition direct assays for the dehydrase enzyme were performed (Table II).

TABLE I

EFFECT OF VALINE-RELATED COMPOUNDS ON THE GROWTH RATE OF WEANLING RATS

Supplement to the basal diet	*Weight gain (g/day)*
None	— 0.64
DL-Valine, 2.00%	+ 2.87
DL-α,β-Dihydroxyisovaleric acid, 2.29%	— 0.61
α-Ketoisovaleric acid, 1.18%	+ 1.56

TABLE II

DEHYDRASE ASSAYS

The complete dehydrase assay system contained 0.05 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.4, 0.02 *M* $MgCl_2$, 0.02 *M* DL-α,β-dihydroxyisovalerate and 0.10–0.30 ml of tissue extract in a final volume of 1.0 ml. These components were incubated in triplicate for 30 min in a nitrogen atmosphere with constant shaking in a water bath at 37°. The keto acid measurement and assay corrections were described previously[5].

Tissue source	*Dehydrase activity (μmoles keto acid formed/0.3 ml extract)*	*Specific activity (μmoles keto acid formed/mg protein)*
Rat-liver homogenate	0.00	0.000
Rat-kidney homogenate	0.00	0.000
Lactobacillus casei	0.00	0.000
Lactobacillus arabinosus	0.01	0.002
Saccharomyces cerevisiae	0.94	0.364
Torulopsis utilis	0.81	0.474
Serratia marcesens	0.72	0.171
Bacterium cadaveris	0.41	0.038
Micrococcus lysodeikticus	0.28	0.130

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[*] A part of this communication was presented at the Fifth International Congress of Nutrition, Washington, D. C., September 1-7, 1960.

Addition of 0.3 ml of a 1:10 rat-liver and -kidney homogenate in 0.25 *M* sucrose failed to catalyze the formation of keto acid under the assay conditions developed for *Saccharomyces cerevisiae*[6]. Incubations at pH 6.8 and 8.0 under nitrogen were also negative.

Table II also contains the dehydrase assay on two groups of micro-organisms. The *Lactobacilli* were obtained by the incubation of inoculum for 16 h at 37° in 400 ml media containing casein hydrolyzate and tryptophan as the nitrogen source. After centrifuging, the 3-6 g of wet packed cells were suspended in 9 vol. cold 0.10 *M* phosphate buffer, pH 7.4, ruptured by a 40 min treatment in a Raytheon 10 Kc ultrasonic oscillator and centrifuged at 18,000 × *g* for 20 min at 3°. Triplicate assay tubes containing 0.3 ml of this supernatant for four different batches of each *Lactobacilli* were essentially negative.

Cell-free extracts of the bottom group (Table II) of 5 microorganisms, obtained from commercial sources, were prepared by similar procedures. Shorter sonic treatment for rupture of the last 4 organisms listed was possible. To maintain a linear response with enzyme concentration, 0.05-0.20 ml of these 5 extracts was used. Correction for the volume of this aliquot was made to facilitate comparisons in Table II.

The above *Lactobacilli*, like the rat, fail to grow with ammonium salts as the nitrogen source. While both can convert α-ketoisovaleric acid to valine due to the presence of transaminase[8], they are devoid of detectable dihydroxy acid dehydrase activity. The last 5 microorganisms listed in Table II, in addition to *Escherichia coli* and *Neurospora crassa*[3], grow on ammonium salts as the sole nitrogen source and can synthesize all of their amino acids. As each contains the dihydroxy acid dehydrase, the two terminal steps of valine formation is similar in these 7 organisms.

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The failure in lipogenesis induced by fasting: a new view

Since the establishment of the fact that fasting almost abolishes hepatic lipogenesis[1,2], investigators have looked for a mechanistic explanation of this inhibition along two lines, a deficiency of either co-factor levels or enzyme levels[3–5]. These two lines of approach have not thus far fully explained the mechanism responsible for suppression of hepatic lipogenesis during fasting. Therefore, this laboratory decided on a new approach and this work has implicated a heretofore unsuspected factor.

A 20 % homogenate of rat liver prepared in 0.25 *M* sucrose was centrifuged for 10 min at 600 × *g* to yield a cell- and nuclei-free supernatant which will be called the "homogenate". A particle-free fraction called the "supernatant" was prepared by centrifuging the homogenate for 1 h at 105,000 × *g*. The incubation system (except that the present system contained ATP (3 m*M*)), the incubation procedures, chemical analyses and ^{14}C analyses are described elsewhere[5].

Liver homogenates prepared from rats fasted for 24 h converted far less [1-^{14}C]-acetate to fatty acids than did those from normal fed rats (Table I). In the case of the fed rats the supernatant incorporated less [1-^{14}C]acetate into fatty acids than did the homogenate from which it came ($P < 0.01$)*; the opposite result was obtained with fasting rats, the supernatant synthesizing more fatty acids than the homogenate ($P < 0.01$)*.

TABLE I

EFFECT OF FASTING ON LIPOGENESIS BY RAT LIVER HOMOGENATE AND SUPERNATANT

Values are means ± standard error of mean.

Physiological state	*Number of rats used*	*Protein content per incubation flask*		*mμmoles [1-^{14}C]acetate converted to fatty acid*	
		Homogenate (mg)	*Supernatant (mg)*	*Homogenate**	*Supernatant**
Fed	6	75 ± 3.4	36 ± 1.4	354 ± 39.3	240 ± 28.2
Fasted	9	79 ± 4.9	38 ± 1.4	93 ± 12.9	147 ± 19.8

* In the case of each rat, homogenate and supernatant fractions were studied simultaneously thus permitting statistical evaluation of the data based on a paired-comparison method[6].

These results suggested that in the liver of the fasted rat, the mitochondria or the microsomes or both suppress the lipogenic activity of the supernatant. To test this possibility further, the effect of each of these particulate fractions on the lipogenic activity of supernatant from fed rats was investigated (Table II). The cytoplasmic particles from fed rats stimulated the lipogenic activity of supernatant from fed rats ($P < 0.02$)* while the cytoplasmic particles from fasted rats inhibited it ($P < 0.01$)*. The mitochondria from both fed and fasted rats depressed lipogenesis by the supernatant from fed rats ($P < 0.01$)*, mitochondria from fasted rats being somewhat more inhibitory. The microsomes from both fed and fasted rats reduced the lipogenic activity of the supernatant from fed rats but the microsomes from the fasted rats had a far greater inhibitory action ($P < 0.001$)*. It should also be noted that the microsomes from fasted rats inhibited supernatant lipogenesis far more than did the mitochondria from these animals ($P < 0.01$)*.

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* *P* values are calculated from paired-comparison statistics[6].

TABLE II

EFFECT OF HEPATIC CYTOPLASMIC PARTICULATE FRACTIONS ON THE LIPOGENIC ACTIVITY OF PARTICLE-FREE HEPATIC SUPERNATANT

Values are means ± standard error of mean

*Liver system**	*mμmoles [1-^{14}C]acetate converted to fatty acids***
"Fed" supernatant	296 ± 25.9
"Fed" supernatant + "fed" cytoplasmic particles	382 ± 24.0
"Fed" supernatant + "fasted" cytoplasmic particles	169 ± 22.8
"Fed" supernatant + "fed" mitochondria	172 ± 14.3
"Fed" supernatant + "fasted" mitochondria	108 ± 10.9
"Fed" supernatant + "fed" microsomes	222 ± 26.8
"Fed" supernatant + "fasted" microsomes	51 ± 8.4

* Cytoplasmic particles were isolated by centrifuging the nuclei-free homogenate at 105,000 × *g* for 60 min. Mitochondria and microsomes were isolated by modification of the method of SCHNEIDER AND HOGEBOOM[7]. The particulate fractions were suspended in a volume of the supernatant fraction equal to that from which they were derived. None of these particulate fractions synthesized fatty acids in the absence of the supernatant fraction.

** The values reported below are average ones for 7 rat pairs. The supernatant plus each of the particulate fraction additions were studied simultaneously for each rat pair, thus permitting statistical evaluation of the data based on a paired-comparison method[6].

It is concluded that the cytoplasmic particles of the liver cell of a fasting rat limit the lipogenic activity of the "particle-free" cytoplasm. The results indicate that the microsomes may be the major, if not the sole, site of this inhibitory action. This possibility is further supported by the finding that sonic treatment of the microsomes releases an inhibitory substance in a water-soluble form. It would seem therefore that, in addition to the possibilities that a deficiency of co-factors or loss of enzymes are responsible for the low rate of hepatic lipogenesis induced by fasting, another mechanism appears probable, the generation of an inhibitor by the microsomes.

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Studies on wheat plants using ^{14}C compounds

XIV. Conversion of [1,7-$^{14}C_2$]-α,α′-diaminopimelic acid to [1-^{14}C] lysine*

Two pathways have been suggested for the biosynthesis of lysine, one involving α-aminoadipic acid[1], and the other involving α,α′-diaminopimelic acid[2,3,4]. Since experiments in this laboratory indicate that [6-^{14}C]-α-aminoadipic acid does not lead to the formation of radioactive lysine in wheat plants[5], it was decided to investigate the possibility that α,α′-diaminopimelic acid[2] is converted to lysine in wheat plants.

Wheat plants 84 days old were injected with a solution of [1,7-$^{14}C_2$]α,α′-diaminopimelic acid**[6] (0.1 ml, 0.5 mg, 0.2 μC) and the plants were harvested mature 25 days later. The kernel contents were fractionated to give arbitrary fractions designated as starch, gluten, salt-soluble protein, ether-soluble material, and bran[7].

Lysine, proline, glutamic acid, and aspartic acid were isolated from the acid hydrolyzates of the chaff, gluten, and salt-soluble protein by column chromatography using the technique of HIRS, MOORE AND STEIN[8]. The carboxyl carbon of lysine was obtained as CO_2 from the acid hydrolyzates of the chaff and salt-soluble protein by incubation with lysine decarboxylase[9].

Lysine samples recovered from the plants were found to have a much higher specific activity than other amino acids isolated; and indeed its activity was considerably greater than the tissues from which it was obtained (Table I). These data indicate a fairly direct conversion of diaminopimelic acid to lysine. This conclusion is supported by results which show that the ^{14}C is preferentially incorporated as the carboxyl group of lysine. The high specificity of this incorporation is shown further by the calculation that, although lysine is a minor constituent of wheat protein, 42 % of the radioactive carbon in the salt-soluble protein appeared in the carboxyl

TABLE I

^{14}C CONTENT OF AMINO ACIDS ISOLATED

Substance assayed	*Specific activity (μC/mole CO_2)*		
	Gluten	*Salt-soluble protein*	*Chaff*
Original material	0.66	1.38	5.60
Proline	0.40	—	1.20
Glutamic acid	0.40	1.10	1.40
Aspartic acid	0.24	0.30	1.0
Lysine	12.40	17.50	83.50
C-1 of lysine by ninhydrin decarboxylation	59.0		
C-1 of lysine by lysine decarboxylase*	—	81.0	455.0
% of [^{14}C]lysine in carbon-1	79	77	91

* *Bacterium cadaveris* NCTC 6578 decarboxylase kindly supplied by Dr. F. J. SIMPSON; CO_2 released was collected by the Conway microdiffusion method[10].

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* Issued as N.R.C. No. 6044.

** [1,7-$^{14}C_2$]-α,α′-Diaminopimelic acid was synthesized in this laboratory for these experiments and no attempt was made to separate its isomers before its use. Methods for its preparation will be reported elsewhere.

group of lysine. Similarly, lysine carboxyl groups accounted for 10 % of the radioactivity in the chaff.

Since conversion of [1,7-$^{14}C_2$]-α,α'-diaminopimelic acid to lysine by decarboxylation would be expected to give [1-^{14}C]lysine the above results provide convincing evidence that wheat plants are capable of carrying out this decarboxylation.

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Fatty acid synthesis in heart sarcosomes

Incubation of rat- or rabbit-heart sarcosomes with sodium [2-^{14}C]acetate, ATP, CoA, TPNH and an oxidizable substrate of the tricarboxylic acid cycle leads to the incorporation of the acetate into the long-chain fatty acids of the mitochondria (Table I). Practically no ^{14}C was found in the non-saponifiable fraction. The incorporation in the fatty acids is nearly completely dependent upon oxidizable substrate, and is appreciably less in the absence of added TPNH and CoA. Succinate is much more effective than glutamate, although the two substrates consume oxygen at about the same rate. This, together with the fact that Amytal inhibited the incorporation with succinate by 66 % and that malate gave a greater rate of incorporation than succinate suggests that oxaloacetate formed by the oxidation of succinate or malate is involved in the incorporation reaction. Oxaloacetate is also formed during the oxidation of glutamate[1,2] but is rapidly removed by transamination with the glutamate. The highest rate of incorporation was found with citrate (and isocitrate) as substrate. It is possible that oxalosuccinate has a role similar to that of oxaloacetate. Both compounds fulfil the structural requirements, suggested by SWICK AND WOOD[3], for transfer of CO_2

Abbreviations: ATP, adenosine triphosphate; CoA, coenzyme A; TPN^+, TPNH, oxidized and reduced triphosphopyridine nucleotide.

to acetyl-CoA to form malonyl-CoA. In the presence of TPNH, malonyl-CoA can be converted to long-chain fatty acids[4].

Gas chromatography showed that the label was present in a number of long-chain fatty acids. Details will be reported in a later publication.

TABLE I

FATTY ACID SYNTHESIS IN RABBIT-HEART SARCOSOMES

1 ml containing 9.5 m*M* KCl, 3 m*M* ethylenediaminetetraacetate, 50 m*M* potassium phosphate buffer, pH 7.5; 5 m*M* $MgCl_2$, 10 m*M* ATP, 3 m*M* $[2\text{-}^{14}C]$acetate (1 μC), 30 mg sarcosomal protein, and where indicated 30 m*M* hydrogen donor, 0.18 m*M* CoA or 0.2 m*M* TPN^+ + 10 m*M* glucose 6-phosphate + glucose 6-phosphate dehydrogenase, was incubated in a shaking manometer flask containing 10% KOH and filter paper in the centre well, in air at 25° for 30 min (except with malate (10 min), since longer incubations did not lead to increasing incorporation). The reaction was stopped by the addition of 1 ml 20 *N* KOH. 4 ml ethanol was added and the mixture saponified at 90° for 15 min. The mixture was acidified with HCl and twice extracted with pentane. The total organic phase was washed with water, plated and counted in a gas-flow counter with thin end window.

Hydrogen donor	*CoA*	*TPNH*	*mμmoles [2-^{14}C]acetate incorporated/h/mg protein*
None	+	+	0.33
Succinate	+	+	2.90
Succinate	—	+	0.94
Succinate	+	—	1.71
Succinate*	+	+	0.99
Glutamate	+	+	0.51
Malate	+	+	3.45
α-Ketoglutarate	+	+	1.30
Citrate	+	+	6.53

* In the presence of 1.7 m*M* Amytal.

The relationship between the system described in this paper and the anaerobic synthesis of fatty acids by rat-liver mitochondria, recently described by WAKIL *et al.*[5], remains to be determined.

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